



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07H 21/00	A1	(11) International Publication Number: WO 96/40711 (43) International Publication Date: 19 December 1996 (19.12.96)
(21) International Application Number: PCT/US96/09551 (22) International Filing Date: 7 June 1996 (07.06.96) (30) Priority Data: 08/485,611 7 June 1995 (07.06.95) US (71) Applicant: MICROPROBE CORPORATION [US/US]; 1725 220th Street, S.E., No. 104, Bothell, WA 98021 (US). (72) Inventors: MEYER, Rich, B., Jr.; 15533 61st Avenue N.E., Bothell, WA 98011 (US). GAMPER, Howard, B.; 14048 212th Drive N.E., Woodinville, WA 98078 (US). KUTYAVIN, Igor, V.; 16520 North Road #B101, Bothell, WA 98012 (US). GALL, Alexander, A.; 16520 North Road #B205, Bothell, WA 98012 (US). PETRIE, Charles, R.; 18459 196th Place N.E., Woodinville, WA 98072 (US). TABONE, John, C.; 12117 166th Place N.E., Bothell, WA 98011 (US). HURST, Gerald, D.; 39 Painted Sunset, The Woodlands, TX 77380 (US). (74) Agents: KLEIN, Howard, J. et al.; Klein & Szekeres, Suite 700, 4199 Campus Drive, Irvine, CA 92715 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: CROSS-LINKING OLIGONUCLEOTIDES		
(57) Abstract <p>Oligonucleotides (ODNs) include a sequence that is complementary to a target sequence in single stranded RNA, or single or double stranded DNA, and an alkylating function which after hybridization alkylates the target sequence. ODNs adapted for alkylating single stranded RNA, such as messenger RNA, are complementary to the target sequence in the Watson Crick sense. ODNs adapted for alkylating double stranded DNA have at least two alkylating functions and are complementary to the target sequence in the Hoogsteen or reverse Hoogsteen sense. With these ODNs both strands of the target sequence are alkylated. A third class of ODNs have at least approximately 26 nucleotide units in a continuous sequence which are complementary to the target sequence of double stranded DNA, and the alkylating function is covalently attached to a nucleotide unit in the continuous sequence. Alkylation or cross-linking with this class of ODNs occurs in the presence of a recombinase enzyme.</p>		

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CROSS-LINKING OLIGONUCLEOTIDES

BACKGROUND OF THE INVENTION

1. Cross-reference to Related Applications

The present application is

(1) a continuation-in-part of application serial number 08/226,949 filed on June 27, 1994, which is a continuation-in-part of application serial number 08/011,482, filed on January 26, 1993;

(2) a continuation-in-part of application serial number 08/334,490 filed on November 4, 1994, which is a continuation of application serial number 08/049,807 filed on April 20, 1993, which is a continuation of application serial number 07/353,857 filed on May 18, 1989, which is a continuation-in-part of application serial number 250,474 filed on September 28, 1988;

(3) a continuation-in-part of application serial number 08/178,733 filed on January 7, 1994, which is a continuation of application serial number 07/748,138 filed on August 21, 1991, which is a continuation-in-part of application serial number 07/353,857 filed on May 18, 1989, which is a continuation-in-part of application serial number 07/250,474 filed on September 28, 1988.

2. Field of the Invention

The present invention is directed to oligonucleotides which have a cross-linking agent covalently attached to one or more nucleotide units, and are capable of binding either by classic Watson Crick or by Hoogsteen or reverse Hoogsteen pairing to a target sequence of DNA or RNA. The cross-linking oligonucleotides of the present invention can be utilized for therapeutic, diagnostic, DNA mapping and similar investigative or analytical purposes.

3. Description of the Prior Art

Oligodeoxynucleotides (ODNs) have great potential as sequence specific pharmaceutical agents for the inhibition of gene expression. Chemically synthesized ODNs may inhibit the expression of specific gene products through formation of duplexes upon hybridization with

1 complementary messenger RNAs (mRNAs). More specifically, these
2 "antisense" ODNs are believed to inhibit the processing or translation of
3 message primarily through an RNase H-mediated cleavage of the target
4 mRNA sequence. Because of this inhibitory effect, antisense ODNs
5 may be useful as anti-viral, anti-parasitic, and anti-cancer agents.
6 However, "antisense" technology is beset with certain fundamental
7 disadvantages relating, for example, to degradation of antisense ODNs
8 by nuclease enzymes, and uptake (or lack of uptake) by cells. To
9 improve their properties, modified antisense ODNs, such as ODNs with
10 modified backbones (oligonucleoside methylphosphonates and
11 phosphorothioates) have been prepared. It has been found however,
12 that improvement in some properties, such as resistance to nuclease
13 enzymes frequently has deleterious effects on other properties, such as
14 cellular uptake and loss of specificity.

15 Another approach to improve the effectiveness of antisense
16 ODNs involves covalently attaching moieties to the antisense ODNs
17 which moieties interact directly with the target RNA upon hybridization
18 and therefore potentiate the antisense activity of the ODN. Groups
19 employed in this regard are intercalating groups, and groups which
20 covalently link with the target RNA after hybridization.

21 Anti-gene ODNs

22 A variation of the "antisense" approach to rational drug design is
23 termed "anti-gene". Whereas antisense ODNs target single stranded
24 mRNA, anti-gene ODNs hybridize with and are capable of inhibiting
25 the function of double-stranded DNA. More specifically, anti-gene
26 ODNs form sequence-specific triple-stranded complexes with a double
27 stranded DNA target and thus interfere with the replication or
28 transcription of selected target genes. As is known, except for certain
29 RNA viruses and nucleic acid-free viroids, DNA is the repository for all
30 genetic information, including regulatory control sequences and
31 non-expressed genes, such as dormant proviral DNA genomes. In

1 contrast, the target for antisense ODNs, mRNA, represents a very small
2 subset of the information encoded in DNA. Thus, anti-gene ODNs
3 have broader applicability and are potentially more powerful than
4 antisense ODNs that merely inhibit mRNA processing and translation.

5 Anti-gene ODNs in the nuclei of living cells can form
6 sequence-specific complexes with chromosomal DNA. The resultant
7 triplexes have been shown to inhibit restriction and/or transcription of
8 the target double stranded DNA. Based on the known stabilities of the
9 two target nucleic acid species (i.e., DNA and RNA), anti-gene
10 interference with DNA functioning has longer lasting effects than the
11 corresponding antisense inhibition of mRNA function.

12 As noted above, anti-gene therapy may be based on the
13 observation that under certain conditions DNA can form triple-stranded
14 complexes. In these triple-stranded complexes, the third strand resides
15 in the major groove of the Watson-Crick base paired double helix,
16 where it hydrogen bonds to one of the two parental strands. A binding
17 code governs the recognition of base pairs by a third base (see allowed
18 triplets below; Hoogsteen or reverse Hoogsteen pairing). In each case,
19 the third strand base is presented first and is followed by the base pair
20 in the Watson-Crick duplex.

21 allowed triplets: A-A-T G-G-C
22 T-A-T C-G-C

23 Certain limitations of this base pair recognition code are apparent
24 from the allowed triplets. First, there is no capability for the
25 recognition of T-A and C-G base pairs; hence, triple strand formation is
26 restricted to runs of purine bases on one strand and pyrimidine bases on
27 the other strand of the duplex. In other words, the third strand or
28 ODN binds only to one strand of the duplex and can only bind to
29 purines. Second, if cytosine is in the third strand ("C"), it must be
30 protonated to be able to hydrogen bond to the guanine of a G-C base

1 pair. The pKa for protonation of cytosine is 4.6, suggesting that at
2 physiological pH the stability of C-G-C triads is likely to be impaired.
3 Third, in all cases triads are maintained by two hydrogen bonds between
4 the third strand base and the purine residue of the duplex base pair.
5 Hence, triple-stranded complexes are generally less stable than the
6 parental double-stranded DNA, which is maintained by a combination
7 of two (A-T) or three (G-C) hydrogen bonds between purine and
8 pyrimidine pairs. (Watson-Crick motif).

9 An important disadvantage of triple strand formation as discussed
10 above is the relatively slow kinetics of triple strand formation.
11 However, triple strand formation can be catalyzed in cells by
12 recombinase enzymes which are practically ubiquitous in cells and whose
13 existence is well known in the art. In addition to a much faster rate of
14 triple strand formation, recombinase enzyme-catalyzed triple strand
15 formation also provides the advantage of universal sequence recognition
16 (in contrast to the A-T and G-C recognition limitation associated with
17 non-enzyme-mediated triple strand formation). More specifically, the
18 recombinase enzyme-mediated recognition motif recognizes all four
19 base pairs, thereby allowing targeting of any double stranded DNA
20 sequence. Second, the nucleoprotein filament, which is the complex
21 formed between a recombinase enzyme and the single-stranded ODN,
22 searches for target double strand DNA homology much more efficiently
23 than does a small naked anti-gene ODN, thus decreasing the
24 concentration of anti-gene ODN required for efficient triple strand
25 complex formation. Third, due to the hydrogen bonding patterns and
26 the novel helical twist involved in enzyme-mediated recognition, the
27 resultant triple strand complex is stable at physiological pH. Fourth,
28 since the cellular recombinational pathway is being harnessed, the DNA
29 in higher order chromatin structures will be accessible for targeting.

30 A first demonstration of the concept of using sequence-specific,
31 antisense oligonucleotides as regulators of gene expression and as

1 chemotherapeutic agents was described by Zamecnik and Stephenson,
2 Proc. Natl. Acad. Sci. USA, 75:280 (1978). These authors showed that a
3 small antisense oligodeoxynucleotide probe can inhibit replication of
4 Rous Sarcoma Virus in cell culture, and that RSV viral RNA translation
5 is inhibited under these conditions (Stephenson et al., Proc. Natl. Acad.
6 Sci. USA 75:285 (1978)). Zamecnik et al., Proc. Natl. Acad. Sci. USA,
7 83:4143 (1986), have also shown that oligonucleotides complementary to
8 portions of the HIV genome are capable of inhibiting protein expression
9 and virus replication in cell culture. Inhibition of up to 95% was
10 obtained with oligonucleotide concentrations of about 70 μ M.
11 Importantly, they showed with labeled phosphate studies that the
12 oligonucleotides enter cells intact and are reasonably stable to
13 metabolism.

14 The concept of covalently linking an inhibitor molecule to a
15 target (such as binding an ODN to an target sequence with a
16 cross-linking arm,) is related to the pioneering work of B.R. Baker,
17 "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley,
18 New York, (1967), who used what was termed "active-site-directed
19 enzyme inhibitors" in chemotherapeutic applications. The concept of
20 incorporating a crosslink in an oligonucleotide has been sporadically
21 discussed by several authors. For example, Knorre and Vlassov, Prog.
22 Nucl. Acid Res. Mol. Biol., 32:291 (1985), have discussed
23 sequence-directed crosslinking ("complementary addressed
24 modification") using an N-(2-chloroethyl)-N-methylaniline group
25 attached to either the 3'- or 5'-terminus of oligonucleotides.
26 Summerton and Bartlett, J. Mol. Biol., 122:145 (1978) have shown that
27 an 8-atom chain, attached to a cytosine residue at its C-4 position and
28 terminating in the highly reactive bromomethyl ketone group, can
29 crosslink to the N-7 of guanosine. Webb and Matteucci, Nucleic Acids
30 Res., 14:7661 (1986), have prepared oligonucleotides containing a
31 5-methyl-N,N-ethanocytosine base which is capable of slow crosslinking

1 with a complementary strand. In a conceptually related alkylation via a
2 linker arm within a DNA hybrid, Iverson and Dervan, Proc. Natl. Acad.
3 Sci. USA, 85:4615 (1988), have shown opposite strand methylation,
4 triggered by BrCN activation of a methylthio ether, predominately on a
5 guanine base located two pairs from the base bearing the linker.
6 Vlassov et al. in Gene 72 (1988) 313-322, describe sequence specific
7 binding and alkylation of plasmid DNA with oligodeoxynucleotide
8 derivatives containing 2-chloroethyl-N-methyl amino phenyl residues.
9 Similar cross-linking, using different cross-linking agent was described by
10 Shaw et al., J. Am. Chem. Soc. 1991, 113, 7765-7766.

11 Further information pertaining to ODNs, chemically modified
12 ODNs and their ability to affect or inhibit replication or translation of a
13 target sequence of DNA or RNA can be found in European Patent
14 Application No. 86309090.8, PCT publication WO8707611, U.S. Patent
15 No. 4,599,303, EP 0259186, PCT publication WO8503075, German
16 Patent DE3310337, and in the publications Blake et al., Biochemistry
17 24:6139 (1985); Umlauf et al., "Triple-helical DNA Pairing
18 Intermediates Formed by recA Protein", Biol. Chem., 265(28),
19 16898-16912 (1990); and Thuong et al., "Chemical synthesis of natural
20 and modified oligodeoxynucleotides.", Biochimie, 1985, 67, 673-684.

21 DNA mapping

22 In addition to chemotherapy or potential chemotherapy utilizing
23 ODNs or modified ODNs, a broad field has developed in the prior art
24 for DNA mapping (gene mapping), that is, for in vitro determination of
25 DNA sequence or partial DNA sequence. An important step in such
26 DNA sequencing (gene mapping) is the cleavage of the target DNA
27 into smaller fragments. The modified ODNs of the present invention
28 also have utility in this field.

29 **SUMMARY OF THE INVENTION**

30 (1) In one aspect the present invention relates to oligonucleotides
31 (ODNs) which have at least one cross-linking agent covalently attached

1 to the oligonucleotide, either to an internal or to a terminal nucleotide
2 unit, and which have a base sequence sufficiently complementary to a
3 single stranded target sequence so as to sequence specifically form a
4 Watson-Crick bonded complex with the target sequence, and thereafter
5 covalently react with the target sequence. Such ODNs can be used for
6 therapeutic purposes as anti-sense agents (targeting messenger RNA) or
7 as sequence specific probes for diagnostic and analytical purposes.

8 (2) In a second aspect the present invention relates to ODNs
9 which have at least two electrophilic cross linking agents covalently
10 attached to the oligonucleotide, either to an internal or to a terminal
11 nucleotide unit, and which are complementary in the Hoogsteen or
12 reverse Hoogsteen pairing sense to a target sequence in double-stranded
13 DNA. The two cross-linking agents may be attached to two different
14 sites of the ODN. Alternatively, the cross-linking agent which is
15 attached to one site on the ODN has two cross-linking functionalities,
16 and therefore in effect comprises two cross-linking agents. The ODNs
17 constructed in accordance with this aspect of the invention form a
18 sequence specific (in the Hoogsteen or reverse Hoogsteen sense) triple
19 stranded complex with the target sequence of double stranded DNA,
20 and the cross-linking agents covalently react with nucleophilic sites on
21 both strands of the target DNA sequence. ODNs in accordance with
22 this aspect of the invention are useful as anti-gene (chemotherapeutic)
23 agents targeting the DNA of an invading cell, organism or pathogen,
24 such as a virus, fungus, parasite, bacterium or malignant cell. ODNs in
25 accordance with this aspect of the invention are also useful as tools for
26 DNA sequencing, gene mapping and related in vitro analytical and
27 diagnostic procedures. Therefore, the target DNA may also be a gene
28 or other duplex DNA which is to be sequenced ("mapped") or otherwise
29 analyzed or investigated in vitro.

30 (3) In a third aspect, the present invention relates to ODNs which
31 have in a substantially continuous sequence at least approximately 26

1 nucleotide units homologous to a target sequence in double stranded
2 DNA. (Those skilled in the art will readily understand that the
3 sequence of the ODN which is homologous to a target sequence of one
4 strand of double stranded DNA is also complementary in the Watson
5 Crick sense to the second strand of the same target sequence in the
6 DNA.) The ODNs in accordance with this aspect of the invention have
7 one or more electrophilic cross linking agents covalently attached to the
8 oligonucleotide, either to an internal or to a terminal nucleotide unit.
9 In vitro, and in the presence of a recombinase enzyme these ODNs are
10 capable of forming sequence specific complexes with the target
11 sequence of double stranded DNA based upon the full "four letter
12 code" Watson Crick type recognition motif, and cross-link with at least
13 one strand of the DNA. In vivo, due to the presence of recombinase
14 enzyme in cells, the ODNs in accordance with this aspect of the
15 invention also form complexes with the target sequence of double
16 stranded DNA and cross-link with at least one strand of the target. In
17 accordance with this aspect of the invention the cross-linking function is
18 preferably attached to a nucleotide unit which is internal in the ODN.

19 The cross-linking function typically includes a linker arm (such as
20 an alkyl, alkoxy, aminoalkyl or amidoalkyl chain) and an electrophilic
21 reactive group which, after complexing with the target sequence of DNA
22 or mRNA is capable of reacting with the target DNA to form a covalent
23 bond therewith. As a result of the covalent bond formation between
24 the modified ODN and the target sequence, replication and/or
25 expression of the target sequence is inhibited, or in diagnostic or
26 mapping application the target is "labeled", or a site for cleavage is
27 created.

28 The ODNs of the present invention, in addition to having a
29 covalently attached cross-linking agent, may also have other
30 modifications, such as modifications of the heterocyclic bases, of the
31 sugar as well as of the phosphate moieties, relative to naturally

1 occurring ribonucleotides and deoxyribonucleotides. The cross-linking
2 agents may be attached to either the heterocyclic bases, to the sugars or
3 modified sugars, or to the phosphate or modified phosphate moieties.

4 DETAILED DESCRIPTION OF THE INVENTION

5 General Embodiments

6 As is known in the art, oligonucleotides (ODNs) comprise a chain
7 of nucleotides which are linked to one another by phosphate ester
8 linkages. Each nucleotide typically comprises a heterocyclic base
9 (nucleic acid base), a sugar moiety attached to the heterocyclic base,
10 and a phosphate moiety which esterifies a hydroxyl function of the sugar
11 moiety. The principal naturally occurring nucleotides include uracil, or
12 thymine, cytosine, adenine and guanine as the heterocyclic bases, and
13 ribose or deoxyribose as the sugar moiety. The foregoing brief summary
14 of basic nucleotide and oligonucleotide structural chemistry is
15 mentioned because, in accordance with the broad principles of the
16 present invention, at least one chemical cross-linking agent group is
17 attached to an oligonucleotide which is complementary to a target
18 sequence of RNA, single or double stranded DNA, as explained in
19 detail below.

20 The oligonucleotides of the invention may comprise
21 ribonucleotides (containing ribose as the only or principal sugar
22 component), deoxyribonucleotides (containing deoxyribose as the
23 principal sugar component), or in accordance with established
24 state-of-the-art modified sugars or sugar analogs may be incorporated in
25 the ODN of the present invention. Thus, in addition to ribose and
26 deoxyribose, the sugar moiety may be pentose, deoxypentose, hexose,
27 deoxyhexose, glucose, arabinose, xylose, lyxose, and a sugar "analog"
28 cyclopentyl group. The sugar may be in a pyranosyl or in a furanosyl
29 form. In the modified ODNs of the present invention the sugar moiety
30 is preferably the furanoside of ribose, deoxyribose, arabinose,
31 2-fluoro-2-deoxyribose or 2-O-methylribose, and the sugar may be

1 attached to the respective heterocyclic bases either in α or β anomeric
2 configuration. The preparation of these sugars or sugar analogs and of
3 the respective "nucleosides" wherein such sugars or analogs are attached
4 to a heterocyclic base (nucleic acid base) per se is known, and need not
5 be described here, except to the extent such preparation is provided here
6 in connection with one or more specific examples. Preferably the sugar
7 moiety is ribofuranose, 2-deoxyribofuranose or
8 2-fluoro-2-deoxyribofuranose in the β configuration.

9 The phosphorous derivative (or modified phosphate group) which
10 may be attached to the sugar or sugar analog moiety in the modified
11 oligonucleotides of the present invention may be a monophosphate,
12 alkylphosphate, alkanephosphate, phosphorothioate, phosphorodithioate
13 or the like. The preparation of the above-noted phosphate analogs, and
14 their incorporation into nucleotides, modified nucleotides and ODNs,
15 per se, is also known and need not be described here. Preferably, the
16 phosphate derivative incorporated into the therapeutic oligonucleotides
17 of the present invention is a "simple" phosphate, which in an
18 internucleotidic bond forms a phosphate diester, and which at the 3' and
19 5' ends of the modified ODNs of the invention may carry the
20 cross-linking agent. In this regard it is noted that recombinase enzymes
21 recognize such "simple" phosphates and deoxyribose backbones. The
22 cross-linking agent is described in substantial detail below.

23 The heterocyclic bases, or nucleic acid bases which are
24 incorporated in the modified ODNs of the present invention may be the
25 naturally occurring principal purine and pyrimidine bases, (namely
26 uracil, or thymine, cytosine, adenine and guanine, as mentioned above),
27 as well as naturally occurring and synthetic modifications of said
28 principal bases. Those skilled in the art will recognize that a large
29 number of "synthetic" non-natural nucleosides comprising various
30 heterocyclic bases and various sugar moieties (and sugar analogs) have
31 become available in the prior art, and that as long as other criteria of

1 the present invention (such as being "complementary" to a target
2 sequence of RNA or DNA, as applicable, in the Watson Crick,
3 Hoogsteen or reverse Hoogsteen sense, as applicable) are satisfied, the
4 novel ODNs of the invention may include one or several heterocyclic
5 bases other than the principal five base components of naturally
6 occurring nucleic acids. Preferably, however, the heterocyclic base in
7 the modified ODNs of the present invention is selected from uracil-5-yl,
8 cytosin-5-yl, adenin-2-yl, adenin-7-yl, adenin-8-yl, guanin-7-yl,
9 guanin-8-yl, 4-aminopyrrolo[2,3-d]pyrimidin-5-yl,
10 2-amino-4-oxopyrrolo[2,3-d]pyrimidin-5-yl,
11 4-aminopyrazolo[3,4-d]pyrimidin-3-yl or
12 4-amino-6-oxopyrazolo[3,4-d]pyrimidin-3-yl groups, where the purines
13 are attached to the sugar moiety of the oligonucleotides via the
14 9-position, the pyrimidines via the 1-position, the pyrrolopyrimidines via
15 the 7-position and the pyrazolopyrimidines via the 1-position.

16 The cross-linking agents incorporated in the present invention
17 need to meet the requirements that (1) each cross-linking agent must be
18 covalently bonded to a site on the ODN, (2) its length and steric
19 orientation must be such that it can reach a suitable reaction site in the
20 target sequence after the ODN is hybridized or complexed with the
21 target (with or without the assistance of an enzyme) (3) and must have
22 a reactive group which will react with a reactive group of the target
23 sequence. As noted above, the cross-linking agents may be covalently
24 attached to the heterocyclic bases, to the sugar or modified sugar
25 residues, or to the phosphate or modified phosphate functions of the
26 ODNs. Any covalent attachment of the cross-linking agent to the ODN
27 and any combination of covalent attachment of two or more
28 cross-linking agents to the ODN is within the broad scope of the
29 present invention.

30 In the simplest terms the cross-linking agent itself may
31 conceptually be divided into two groups or moieties, namely the reactive

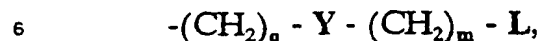
1 group, which is typically and preferably an electrophilic leaving group
2 (L), and an "arm" (A) which attaches the leaving group L to the
3 respective site on the ODN. The leaving group L may be chosen from,
4 for example, such groups as chloro, bromo, iodo, $\text{SO}_2\text{R}''$, or $\text{S}^+\text{R}'''\text{R}''''$,
5 where each of R'' and R''' is independently C_{1-6} alkyl or aryl or R'' and
6 R''' together form a C_{1-6} alkylene bridge. Chloro, bromo and iodo are
7 preferred. Within these groups haloacetyl groups such as $-\text{COCH}_2\text{I}$, and
8 bifunctional "nitrogen mustards", such as $-\text{N}-[(\text{CH}_2)_2-\text{Cl}]_2$ are preferred.
9 The leaving group will be altered by its leaving ability. Depending on
10 the nature and reactivity of the particular leaving group, the group to be
11 used is chosen in each case to give the desired specificity of the
12 irreversibly binding probes.

13 Although as noted above the "arm" (or linker arm) A may
14 conceptually be regarded as a single entity which covalently bonds the
15 ODN to the leaving group L, and maintains the leaving group L at a
16 desired distance and steric position relative to the ODN, in practice the
17 "arm" A may be constructed in a synthetic scheme where a bifunctional
18 molecule is covalently linked to the ODN (for example by a phosphate
19 ester bond to the 3' or 5' terminus, or by a carbon-to-carbon bond to a
20 heterocyclic base) through its first functionality, and is also covalently
21 linked through its second functionality (for example an amine) to a
22 "hydrocarbyl bridge" (alkyl bridge, alkylaryl bridge or aryl bridge, or the
23 like) which, in turn, carries the leaving group.

24 A general formula of the cross linking function is thus $-\text{A}-\text{L}$, or
25 $-\text{A}-\text{L}_2$, where L is the above defined leaving group and A is a moiety that
26 is covalently linked to the ODN. The A "arm" moiety itself should be
27 unreactive (other than through the leaving group L) under the
28 conditions of hybridization of the ODN with the target DNA sequence,
29 and should maintain the leaving group L in a desired steric position and
30 distance from the desired site of reactions such as an N-7 position of a

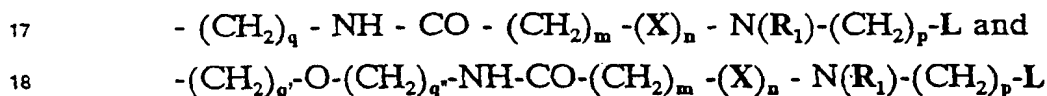
1 guanosine residue in the target sequence. Generally speaking, the
2 length of the A group should be equivalent to the length of a normal
3 alkyl chain of approximately 2 to 50 carbons.

4 An exemplary more specific formula for a class of preferred
5 embodiments of the cross-linking function is



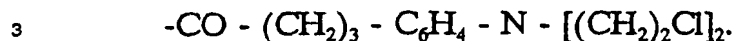
7 where L is the leaving group, defined above, each of m and q is
8 independently 0 to 8, inclusive, and where Y is defined as a "functional
9 linking group". A "functional linking group" is a group that has two
10 functionalities, for example -NH₂ and -OH, or -COOH and -OH, or
11 -COOH and -NH₂, which are capable of linking the (CH₂)_q and (CH₂)_m
12 bridges. An acetylenic terminus (HC≡C-) is also a suitable functionality
13 as a precursor for Y, because it can be coupled to certain heterocycles
14 and thereafter hydrogenated, as described below.

15 Other exemplary and more specific formulas for a class of
16 preferred embodiments of the cross-linking function are



19 where q, m and L are defined as above, q' is 3 to 7 inclusive, q" is
20 1 to 7 inclusive, X is phenyl or simple substituted phenyl (such as
21 chloro, bromo, lower alkyl or lower alkoxy substituted phenyl), n is 0 or
22 1, p is an integer from 1 to 6, and R₁ is H, lower alkyl or (CH₂)_p-L.
23 Preferably p is 2. Those skilled in the art will recognize that the
24 structure - N(R₁)-(CH₂)₂-L describes a "nitrogen mustard", which is a
25 class of potent alkylating agents. Particularly preferred within the scope
26 of the present invention are those modified ODNs where the
27 cross-linking agent includes the functionality - N(R₁)-(CH₂)₂-L where L
28 is halogen, preferably chlorine; and even more preferred are those
29 modified ODNs where the cross linking agent includes the grouping -
30 N-[(CH₂)₂-L]₂ (a "bifunctional" N-mustard).

1 A particularly preferred partial structure of the cross linking
2 agent includes the grouping



4 In a particularly preferred embodiment the just-noted cross-linking
5 group is attached to an *n*-hexylamine bearing tail at the 5' and 3' ends
6 of the ODN in accordance with the following structure:

7 $\text{R}'\text{-O}-(\text{CH}_2)_6\text{-NH} - \text{CO} - (\text{CH}_2)_3 - \text{C}_6\text{H}_4 - \text{N} - [(\text{CH}_2)_2\text{Cl}]_2$ where R'
8 signifies the terminal 5' or 3'-phosphate group of the ODN.

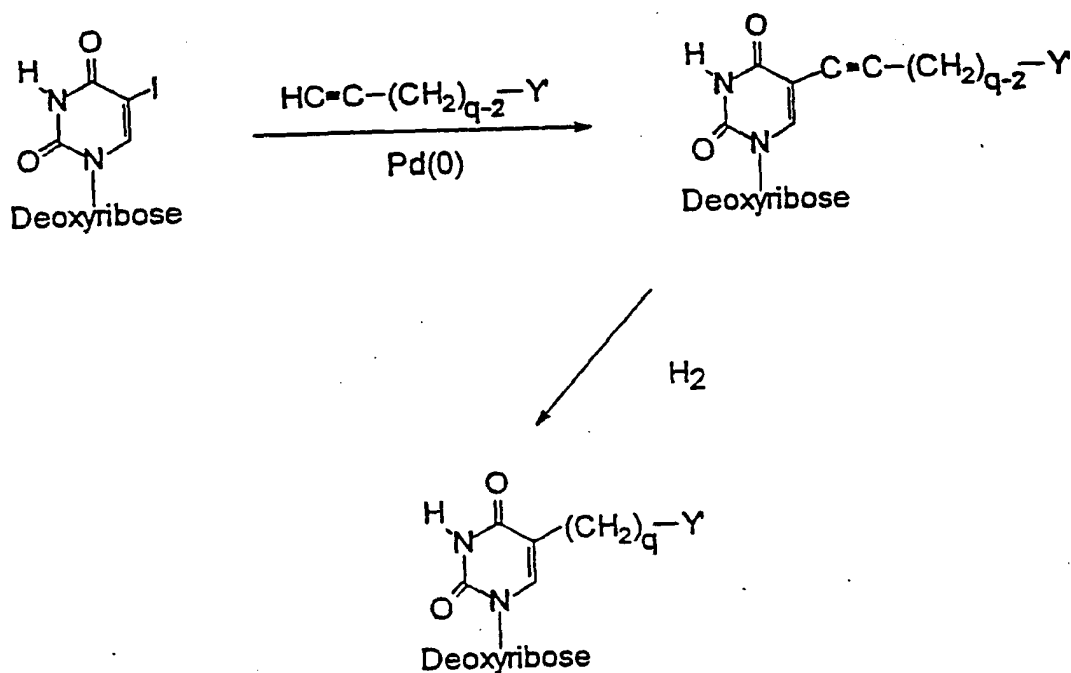
9 Other examples for the A-L group, particularly when attached to
10 a heterocyclic base in the oligonucleotide (such as to the 5-position of
11 2'-deoxyuridine) are 3-iodoacetamidopropyl,
12 3-(4-bromobutyramido)propyl, 4-iodoacetamidobutyl and
13 4-(4-bromobutyramido)butyl groups.

14 In accordance with other preferred embodiments, the
15 cross-linking functionality is covalently linked to the heterocyclic base,
16 for example to the uracil moiety of a 2'-deoxyuridylic acid building
17 block of the ODN. The linkage can occur through the intermediacy of
18 an amino group, that is, the "arm-leaving group combination" (A-L) may
19 be attached to a 5-amino-2'-deoxyuridylic acid building unit of the
20 ODN. In still other preferred embodiments the "arm-leaving group
21 combination" (A-L) is attached to the 5-position of the 2'-deoxyuridylic
22 acid building unit of the ODN by a carbon-to-carbon bond. Generally
23 speaking, 5-substituted-2'-deoxyuridines can be obtained by an
24 adaptation of the general procedure of Robins et al. (Can. J. Chem.,
25 60:554 (1982); J. Org. Chem., 48:1854 (1983)), as shown in **Reaction**
26 **Scheme 1**. In accordance with this adaptation, the palladium-mediated
27 coupling of a substituted 1-alkyne to 5-iodo-2'-deoxyuridine gives an
28 acetylene-coupled product. The acetylenic dUrd analog is reduced, with
29 Raney nickel for example, to give the saturated compound, which is
30 then used for direct conversion to a reagent for use on an automated

1 DNA synthesizer, as described below. In **Reaction Scheme 1,q** is
2 defined as above, and **Y'** is either **Y** (as defined above) or is a suitable
3 protected derivative of **Y**. **Y'** can also be defined as a group which
4 terminates in a suitably protected nucleophilic function, such as a
5 protected amine. Examples of reagents which can be coupled to
6 5-iodo-2'-deoxyuridine in accordance with this scheme are
7 $\text{HC}\equiv\text{CCH}_2\text{OCH}_2\text{CH}_2\text{N}(\text{CO})_2\text{C}_6\text{H}_4$ (phtalimidoethoxypropyne),
8 $\text{HC}\equiv\text{CCH}_2\text{OCH}_2\text{CH}_2\text{NHCOCF}_3$ (trifluoroacetamidoethoxypropyne),
9 $\text{HC}\equiv\text{CCH}_2\text{N}(\text{CO})_2\text{C}_6\text{H}_4$ (phtalimidopropyne) and
10 $\text{HC}\equiv\text{CCH}_2\text{NHCOCF}_3$ (trifluoroacetamidopropyne),

11 In these examples the nucleosides which are obtained in this
12 scheme are incorporated into the desired ODN, and the alkylating
13 portion of the cross-linking agent is attached to the terminal amino
14 group of "**Y'**" only after removal of the respective phtalic or
15 trifluoroacetyl blocking groups.

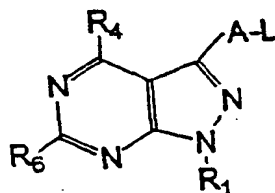
16 Another particularly preferred example of an "arm-leaving group
17 combination" (**A-L**) is attachment of a nitrogen-mustard type alkylating
18 agent (or other alkylating agent) to the amino function of a
19 5-(3-aminopropyl)-2'-deoxyuridine building unit of the ODN. The
20 appropriate nucleotide building unit for ODN synthesis which includes
21 the 5-(3-aminopropyl)-2'-deoxyuridine nucleoside moiety can be
22 obtained in analogy to **Reaction Scheme 1**, and in accordance with the
23 teaching of Meyer et al., J. Am. Chem. Soc. 1989, 111, 8517. In this
24 particularly preferred embodiment the nucleotide having the
25 5-(3-aminopropyl)-2'-deoxyuridine moiety is incorporated into the ODN
26 by routine synthesis, and the cross-linking function is introduced by
27 reacting the ODN with an activated form of a "nitrogen mustard", such
28 as 2,3,5,6-tetrafluorophenyl-4'-[bis(2-chloroethyl)amino]phenylbutyrate
29 (Chlorambucil 2,3,5,6-tetrafluorophenyl ester; chlorambucil itself is
30 commercially available).



Reaction Scheme 1 Other examples of

nucleotides where the crosslinking agent is attached to a heterocyclic base, are 2'-deoxy-4-aminopyrazolo[3,4-d]pyrimidine derivatives. The general structure of these derivatives is shown below in Formula 1. A-L represents the "arm" and the "leaving group" of the cross-linking functionality, as described above. R_1 represents the sugar moiety as described above, and R_4 and R_6 independently are H, OR, SR, NHOR, NH_2 or $\text{NH}(\text{CH}_2)_t\text{NH}_2$, where R is H or C_{1-6} alkyl, t is 0 to 12. These compounds can be made from 3,4-disubstituted and 3,4,6-trisubstituted pyrazolo[3,4-d]pyrimidines, in accordance with the teaching of

1 Kobayashi in Chem. Phar. Bull. 21:941-951 (1973) which is incorporated
2 herein by reference.



Formula 1

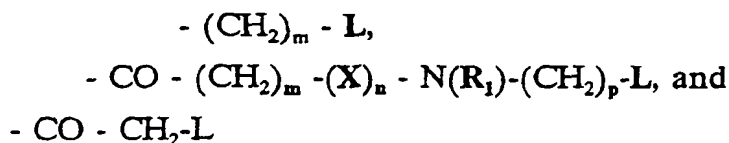
14 Discussing still in general terms the structures of the modified
15 ODNs of the present invention, it is noted that examination of
16 double-stranded DNA by ball-and-stick models and high resolution
17 computer graphics indicates that the 7-position of the purines and the
18 5-position of the pyrimidines lie in the major groove of the B-form
19 duplex of double-stranded nucleic acids. These positions can be
20 substituted with side chains of considerable bulk without interfering with
21 the hybridization properties of the bases. These side arms may be
22 introduced either by derivatization of dThd or dCyd, or by
23 straightforward total synthesis of the heterocyclic base, followed by
24 glycosylation. These modified nucleosides may be converted into the
25 appropriate activated nucleotides for incorporation into oligonucleotides
26 with an automated DNA synthesizer. With the
27 pyrazolo[3,4-*d*]pyrimidines, which are analogs of adenine, the
28 crosslinking arm is attached at the 3-position, which is equivalent to the
29 7-position of purine.

30 The crosslinking side chain (arm = A) should be of sufficient
31 length to reach across the major groove from a purine 7- or 8-position,

pyrimidine 5-position, pyrrolopyrimidine 5-position or
 pyrazolopyrimidine 3-position and react with the N-7 of a purine
 (preferably guanine) located above (on the oligomer 3'-side) the base
 pair containing the modified analog.

The crosslinking side chain (arm = A) holds the functional group away
 from the base when the base is paired with another within the
 double-stranded complex. As noted above, broadly the arm A should be
 equivalent in length to a normal alkyl chain of 2 to 50 carbons.

Preferably, the arms include alkylene groups of 1 to 12 carbon atoms,
 alkenylene groups of 2 to 12 carbon atoms and 1 or 2 olefinic bonds,
 alkynylene groups of 2 to 12 carbon atoms and 1 or 2 acetylenic bonds,
 or such groups substituted at a terminal point with nucleophilic groups
 such as oxy, thio, amino or chemically blocked derivatives thereof (e.g.,
 trifluoroacetamido, phthalimido, CONR', NR'CO, and SO₂NR', where
 R' = H or C₁₋₆alkyl). Such functionalities, including aliphatic or
 aromatic amines, exhibit nucleophilic properties and are capable of
 serving as a point of attachment to such groups as



which are described above as components of exemplary cross-linking
 functional groups.

After the nucleoside or nucleotide unit which carries the
 crosslinking functionality A-L, or a suitable precursor thereof, (such as
 the - (CH₂)_q - NH₂ or - (CH₂)_q - Y group, where Y terminates with a
 nucleophilic group such as NH₂) is prepared, further preparation of the
 modified oligonucleotides of the present invention can proceed in
 accordance with state-of-the-art. Thus, to prepare oligonucleotides,
 protective groups are introduced onto the nucleosides or nucleotides
 and the compounds are activated for use in the synthesis of

oligonucleotides. The conversion to protected, activated forms follows the procedures as described for 2'-deoxynucleosides in detail in several reviews. See, Sonveaux, Bioorganic Chemistry, 14:274-325 (1986); Jones, in "Oligonucleotide Synthesis, a Practical Approach", M.J. Gait, Ed., IRL Press, p. 23-34 (1984).

The activated nucleotides are incorporated into oligonucleotides in a manner analogous to that for DNA and RNA nucleotides, in that the correct nucleotides will be sequentially linked to form a chain of nucleotides which is complementary to a sequence of nucleotides in target DNA or RNA. The nucleotides may be incorporated either enzymatically or via chemical synthesis. The nucleotides may be converted to their

5'-O-dimethoxytrityl-3'-(N,N-diisopropyl)phosphoramidite cyanoethyl ester derivatives, and incorporated into synthetic oligonucleotides following the procedures in "Oligonucleotide Synthesis: A Practical Approach", supra. The N-protecting groups are then removed, along with the other oligonucleotide blocking groups, by post-synthesis aminolysis, by procedures generally known in the art.

In a preferred embodiment, the activated nucleotides may be used directly on an automated DNA synthesizer according to the procedures and instructions of the particular synthesizer employed. The oligonucleotides may be prepared on the synthesizer using the standard commercial phosphoramidite or H-phosphonate chemistries.

A moiety containing the leaving group, such as a haloacyl group ($\text{CO}-\text{CH}_2-\text{L}$ where L is halogen for example I) or $-\text{CO}-(\text{CH}_2)_m-(\text{X})_n-$ $\text{N}(\text{R}_1)-(\text{CH}_2)_p-\text{L}$ group (even more preferably a $\text{CO}-(\text{CH}_2)_3-\text{C}_6\text{H}_4-\text{N}-[\text{CH}_2\text{CH}_2\text{Cl}]_2$) may be added to the aminoalkyl or like groups $(-\text{CH}_2)_q-\text{Y}$ following incorporation into oligonucleotides and removal of any blocking groups. For example, addition of an α -haloacetamide may be verified by a changed mobility of the modified

1 compound on HPLC, corresponding to the removal of the positive
2 charge of the amino group, and by subsequent readdition of a positive
3 charge by reaction with 2-aminoethanethiol to give a derivative with
4 reverse phase HPLC mobility similar to the original
5 aminoalkyl-oligonucleotide.

6 In the situations where the cross linking agent (A-L moiety) is
7 attached to the 3' or 5' terminus of the oligonucleotide, for example by
8 an alkylamine linkage of the formula $-(CH_2)_q-Y$ (Y terminating in an
9 amine), the oligonucleotide synthesis may be performed to first yield the
10 oligonucleotide with said aminoalkyl tail, to which then an alkylating
11 moiety, such as the above-noted haloacylgroup (CO-CH₂-L) or - CO -
12 $(CH_2)_m-(X)_n-N(R_1)-(CH_2)_p-L$ is introduced.

13 Generally speaking the oligonucleotides of the invention may
14 include up to approximately 3000 nucleotide units, although shorter
15 oligonucleotides are preferred, as described below.

16 **CROSS-LINKING OLIGONUCLEOTIDES FOR ANTI-SENSE**
17 **THERAPY AND APPLICATION AS PROBES FOR SINGLE**
18 **STRANDED DNA AND RNA**

19 In accordance with the first broad aspect of the invention the
20 ODN of the invention is used to hybridize with and cross-link with
21 single stranded RNA, such as messenger RNA, or single stranded DNA.
22 Duplex formation and cross-linking with messenger RNA can serve
23 therapeutic purposes (anti-sense) in that by incapacitating the messenger
24 RNA it inhibits gene expression resulting in protein synthesis.
25 Hybridization and cross linking in an in vitro system can serve for
26 diagnostic and analytical purposes. In each instance of utilization of the
27 ODNs in accordance with this aspect of the invention, the ODN has a
28 nucleotide sequence which is complementary (or substantially
29 complementary) in the Watson Crick sense to the target sequence in the
30 single stranded RNA or single stranded DNA, and at least one

1 covalently attached cross-linking agent. Further description of utilizing
2 the ODNs of the present invention as hybridization probes and evidence
3 of sequence specific cross-linking to single stranded DNA (fragments of
4 human papilloma virus (HPV) and human cytomegalovirus (HCV)
5 sequence) and related experimental details are given below.

6 Probes may be labeled by any one of several methods typically
7 used in the art. A common method of detection is the use of
8 autoradiography with ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P labeled probes or the like.
9 Other reporter groups include ligands which bind to antibodies labeled
10 with fluorophores, chemiluminescent agents, and enzymes.

11 Alternatively, probes can be conjugated directly with labels such as
12 fluorophores, chemiluminescent agents, enzymes and enzyme substrates.
13 Alternatively, the same components may be indirectly bonded through a
14 ligand-antiligand complex, such as antibodies reactive with a ligand
15 conjugated with label. The choice of label depends on sensitivity
16 required, ease of conjugation with the probe, stability requirements, and
17 available instrumentation.

18 The choice of label dictates the manner in which the label is
19 incorporated into the probe. Radioactive probes are typically made
20 using commercially available nucleotides containing the desired
21 radioactive isotope. The radioactive nucleotides can be incorporated
22 into probes, for example, by using DNA synthesizers, by
23 nick-translation, by tailing of radioactive bases in the 3' end of probes
24 with terminal transferase, by copying M13 plasmids having specific
25 inserts with the Klenow fragment of DNA polymerase in the presence
26 of radioactive dNTP's, or by transcribing RNA from templates using
27 RNA polymerase in the presence of radioactive rNTP's.

28 Non-radioactive probes can be labeled directly with a signal (e.g.,
29 fluorophore, chemiluminescent agent or enzyme) or labeled indirectly by
30 conjugation with a ligand. For example, a ligand molecule is covalently
31 bound to the probe. This ligand then binds to a receptor molecule

1 which is either inherently detectable or covalently bound to a detectable
2 signal, such as an enzyme or photoreactive compound. Ligands and
3 antiligands may be varied widely. Where a ligand has a natural
4 "antiligand", namely ligands such as biotin, thyroxine, and cortisol, it can
5 be used in conjunction with its labeled, naturally occurring antiligand.
6 Alternatively, any haptenic or antigenic compound can be used in
7 combination with a suitably labeled antibody. A preferred labeling
8 method utilizes biotin-labeled analogs of oligonucleotides, as disclosed
9 in Langer et al., Proc. Natl. Acad. Sci. USA 78:6633-6637 (1981), which
10 is incorporated herein by reference.

11 Enzymes of interest as reporter groups will primarily be
12 hydrolases, particularly phosphatases, esterases, ureases and
13 glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent
14 compounds include fluorescein and its derivatives, rhodamine and its
15 derivatives, dansyl, umbelliferone, rare earths, etc. Chemiluminescers
16 include luciferin, acridinium esters and 2,3-dihydrophthalazinediones,
17 e.g., luminol.

18 The specific hybridization conditions are not critical and will vary
19 in accordance with the investigator's preferences and needs. Various
20 hybridization solutions may be employed, comprising from about 20% to
21 about 60% volume, preferably about 30%, of a polar organic solvent. A
22 common hybridization solution employs about 30-60% v/v formamide,
23 about 0.5 to 1M sodium chloride, about 0.05 to 0.1M buffers, such as
24 sodium citrate, Tris HCl, PIPES or HEPES, about 0.05% to 0.5%
25 detergent, such as sodium dodecylsulfate, and between 1-10 mM EDTA,
26 0.01% to 5% ficoll (about 300-500 kdal), 0.1% to 5%
27 polyvinyl-pyrrolidone (about 250-500 kdal), and 0.01 % to 10% bovine
28 serum albumin. Also included in the typical hybridization solution will
29 be unlabeled carrier nucleic acids from about 0.1 to 5 mg/ml, e.g.,
30 partially fragmented calf thymus or salmon sperm DNA, and/or partially
31 fragmented yeast RNA and optionally from about 0.5% to 2% wt./vol.

glycine. Other additives may also be included, such as volume exclusion agents which include a variety of polar water-soluble or swellable agents, such as anionic polyacrylate or polymethylacrylate, and charged saccharidic polymers, such as dextran sulfate.

The particular hybridization technique is not essential to the invention. Hybridization techniques are generally described in "Nucleic Acid Hybridization, A Practical Approach", Hames and Higgins, Eds., IRL Press, 1985; Gall and Pardue, Proc. Natl. Acad. Sci., U.S.A., 63:378-383 (1969); and John et al., Nature, 223:582-587 (1969). As improvements are made in hybridization techniques, they can readily be applied.

The amount of labeled probe which is present in the hybridization solution may vary widely. Generally, substantial excess of probe over the stoichiometric amount of the target nucleic acid will be employed to enhance the rate of binding of the probe to the target DNA or RNA.

Various degrees of stringency of hybridization can be employed. As the conditions for hybridization become more stringent, there must be a greater degree of complementarity between the probe and the target for the formation of a stable duplex. The degree of stringency can be controlled by temperature, ionic strength, the inclusion of polar organic solvents, and the like. For example, temperatures employed will normally be in the range of about 20° to 80°C, usually 25° to 75°C. For probes of 15-50 nucleotides in 50% formamide, the optimal temperature range can vary from 22-65°C. With routine experimentation, one can define conditions which permit satisfactory hybridization at room temperature. The stringency of hybridization is also conveniently varied by changing the ionic strength and polarity of the reactant solution through manipulation of the concentration of formamide within the range of about 20% to about 50%.

Treatment with ultrasound by immersion of the reaction vessel into commercially available sonication baths can oftentimes accelerate

1 the hybridization rates.

2 After hybridization at a temperature and time period appropriate
3 for the particular hybridization solution used, the glass, plastic, or filter
4 support to which the probe-target hybrid is attached is introduced into a
5 wash solution typically containing similar reagents (e.g., sodium chloride,
6 buffers, organic solvents and detergent), as provided in the hybridization
7 solution. These reagents may be at similar concentrations as the
8 hybridization medium, but often they are at lower concentrations when
9 more stringent washing conditions are desired. The time period for
10 which the support is maintained in the wash solutions may vary from
11 minutes to several hours or more.

12 Either the hybridization or the wash medium can be stringent.
13 After appropriate stringent washing, the correct hybridization complex
14 may now be detected in accordance with the nature of the label.

15 The probe may be conjugated directly with the label. For
16 example, where the label is radioactive, the support surface with
17 associated hybridization complex substrate is exposed to X-ray film.
18 Where the label is fluorescent, the sample is detected by first irradiating
19 it with light of a particular wavelength. The sample absorbs this light
20 and then emits light of a different wavelength which is picked up by a
21 detector ("Physical Biochemistry", Freifelder, D., W. H. Freeman & Co.,
22 1982, pp. 537-542). Where the label is an enzyme, the sample is
23 detected by incubation with an appropriate substrate for the enzyme.
24 The signal generated may be a colored precipitate, a colored or
25 fluorescent soluble material, or photons generated by bioluminescence
26 or chemiluminescence. The preferred label for dipstick assays generates
27 a colored precipitate to indicate a positive reading. For example,
28 alkaline phosphatase will dephosphorylate indoxyl phosphate which then
29 will participate in a reduction reaction to convert tetrazolium salts to
30 highly colored and insoluble formazans.

31 Detection of a hybridization complex may require the binding of

1 a signal generating complex to a duplex of target and probe
2 polynucleotides or nucleic acids. Typically, such binding occurs through
3 ligand and antiligand interactions as between a ligand-conjugated probe
4 and an antiligand conjugated with a signal. The binding of the signal
5 generation complex is also readily amenable to accelerations by
6 exposure to ultrasonic energy.

7 The label may also allow indirect detection of the hybridization
8 complex. For example, where the label is a hapten or antigen, the
9 sample can be detected by using antibodies. In these systems, a signal is
10 generated by attaching fluorescent or enzyme molecules to the
11 antibodies or in some cases, by attachment to a radioactive label.
12 (Tijssen, P., "Practice and Theory of Enzyme Immunoassays, Laboratory
13 Techniques in Biochemistry and Molecular Biology", Burdon, R.H., van
14 Knippenberg, P.H., Eds., Elsevier, 1985, pp. 9-20).

15 The amount of labeled probe present in the hybridization solution
16 may vary widely, depending upon the nature of the label, the amount of
17 the labeled probe that can reasonably bind to the cellular target nucleic
18 acids, and the precise stringency of the hybridization medium and/or
19 wash medium. Generally, substantial probe excesses over the
20 stoichiometric amount of the target will be employed to enhance the
21 rate of binding of the probe to the target nucleic acids.

22 This first aspect of the invention is also directed to a method or
23 identifying target single stranded nucleic acid sequences, which method
24 comprises utilizing an oligonucleotide probe including at least one ODN
25 having a cross-linking agent and a label as described above.

26 In one embodiment, the method comprises the steps of:

- 27 (a) denaturing nucleic acids in the sample to be tested;
28 (b) hybridizing to the target nucleic acids an
29 oligonucleotide probe (hereinafter sometimes "probe") including at least
30 one labeled ODN having a cross-linker covalently attached, wherein the
31 ODN comprises a sequence complementary to that of the target nucleic

1 acid sequence;
2 (c) washing the sample to remove unbound probe;
3 (d) incubating the sample with detecting agents; and
4 (e) inspecting the sample. The above method may be
5 conducted following procedures well known in the art.

6 An assay for identifying target single stranded nucleic acid
7 sequences utilizing a labeled oligonucleotide probe including the
8 covalently attached cross-linking agent and comprising the above
9 method is contemplated for carrying out the invention. Such an assay
10 may be provided in kit form. For example, a typical kit includes the
11 probe reagent (ODN) having a sequence complementary to that of the
12 target nucleic acids; a denaturation reagent for converting
13 double-stranded nucleic acid to a single-stranded nucleic acid; and a
14 hybridization reaction mixture. The kit can also include a
15 signal-generating system, such as an enzyme for example, and a
16 substrate for the system.

17 The following examples are provided to illustrate the present
18 invention without limiting same. "RT" means room temperature.

19 General

20 Thin layer chromatography was performed on silica gel 60 F 254
21 plates (Analtech) using the following solvent mixtures: A- 90%
22 methylene chloride:10% methanol; B- 50% ethyl acetate:50% hexanes;
23 C- 70% ethyl acetate: 10% methanol: 10% water:10% acetone; D- 50%
24 ether:50% hexanes. Flash chromatography was performed using 60 F
25 254 silica (Merck). Oligonucleotides were synthesized on an Applied
26 Biosystems Model 380B Synthesizer. Oligonucleotides were isotopically
27 labeled using T4 Polynucleotide kinase (BRL) and γ -³²P-ATP (New
28 England Nuclear).

29 EXAMPLE 1:

30 6-(Tritylamino)caproic Acid.

31 6-Aminocaproic acid (26 g, 0.2 mole) was dissolved in

1 dichloromethane (200 mL) by the addition of triethylamine (100 mL).
2 Trityl chloride (120 g, 0.45 mole) was added and the solution stirred for
3 36 hours. The resulting solution was extracted with 1N HCl and the
4 organic layer evaporated to dryness. The residue was suspended in
5 2-propanol/1N NaOH (300 mL/100 mL) and refluxed for 3 hours. The
6 solution was evaporated to a thick syrup and added to dichloromethane
7 (500 mL). Water was added and acidified. The phases were separated,
8 and the organic layer dried over sodium sulfate and evaporated to
9 dryness. The residue was suspended in hot 2-propanol, cooled, and
10 filtered to give 43.5 (58%) of 6-(tritylamino)caproic acid, useful as an
11 intermediate compound.

12 EXAMPLE 2:

13 5-(Tritylamino)pentylhydroxymethylenemalononitrile.

14 To a dichloromethane solution of 6-(tritylamino)-caproic acid
15 (20.0 g, 53 mmole) and triethylamine (20 mL) in an ice bath was added
16 dropwise over 30 min isobutylchloroformate (8.3 mL, 64 mmole). After
17 the mixture was stirred for 2 hours in an ice bath, freshly distilled
18 malononitrile (4.2 g, 64 mmole) was added all at once. The solution
19 was stirred for 2 hours in an ice bath and for 2 hours at RT. The
20 dichloromethane solution was washed with ice cold 2N HCl (300 mL)
21 and the biphasic mixture was filtered to remove product that
22 precipitated (13.2 g). The phases were separated and the organic layer
23 dried and evaporated to a thick syrup. The syrup was covered with
24 dichloromethane and on standing deposited fine crystals of product.
25 The crystals were filtered and dried to give 6.3 g for a total yield of 19.5
26 g (87%) of the product, which is useful as an intermediate. EXAMPLE
27 3:

28 5-(Tritylamino)pentylmethoxymethylenemalononitrile.

29 A suspension of the malononitrile of Example 2 (13 g, 31 mmole)
30 in ether/dichloromethane (900 mL/100 mL), cooled in an ice bath, was
31 treated with a freshly prepared ethereal solution of diazomethane (from

1 50 mmole of Diazald^R (Aldrich Chemical Company)). The solution was
2 stirred for 6 hours and then neutralized with acetic acid (10 mL). The
3 solution was evaporated to dryness and the residue chromatographed on
4 silica gel using dichloromethane/acetone (4/1) as the eluent. Fractions
5 containing product were pooled and evaporated to a syrup. The syrup
6 was triturated with dichloromethane to induce crystallization. The
7 crystals were filtered and dried to give 8.3 g (61%) of
8 chromatographically pure product, useful as an intermediate compound.

9 EXAMPLE 4:

10 5-Amino-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile.

11 To a methanol solution (100mL) of the product of Example 3
12 (7.0 g, 16 mmole) in an ice bath was added hydrazine monohydrate (7.8
13 mL, 160 mmole) dropwise over 15 min. After stirring for 30 min in an
14 ice bath, the solution was evaporated to dryness. The residue was
15 suspended in cold methanol and filtered to give 7.1 g (100%) of
16 5-amino-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile, useful as an
17 intermediate, after drying. An analytical sample was prepared by
18 recrystallization from water.

19 EXAMPLE 5:

20 5-Amino-1-(2-deoxy-3,5-di-O-toluoyl-β-D-erythropento-
21 furanosyl)-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile.

22 An ice cold solution of the carbonitrile from Example 4 (3.5 g, 8
23 mmole) was treated with sodium hydride and stirred for 30 min at
24 0-4°C. 1-Chloro-1,2-dideoxy-3,5-di-O-toluoylribofuranose was added
25 and the solution stirred for 1 hour at 0-4°C. The solution was poured
26 into a saturated solution of sodium bicarbonate and extracted with
27 dichloromethane. The organic layer was dried over sodium sulfate and
28 evaporated to dryness. The residue was flash chromatographed. The
29 organic layer was dried over sodium sulfate and evaporated to dryness.
30 The residue was flash chromatographed on silica gel using toluene/ethyl
31 acetate (5/1) as eluent. Two major products were isolated and

1 identified as the N-1 and N-2 isomers in 57% (3.6 g) and 20% (1.2 g)
2 N-1 and N-2 yields, respectively. Approximately 1 g of a mixture of N-1
3 and N-2 isomers was also collected. Overall yield of glycosylated
4 material was 5.8 g (92%). The N-1 isomer,
5 5-amino-1-(2-deoxy-3,5-di-o-toluoyl- β -D-erythropentofuranosyl)-3-[(5-trit
6 yl amino)-pentyl]pyrazole-4-carbonitrile, was used without further
7 purification in Example 6.

8 EXAMPLE 6:

9 1-(2-Deoxy- β -D-erythropentofuranosyl)-3-[5-(trityl-
10 amino)-pentyl]pyrazolo[3,4-d]pyrimidin-4-amine.

11 To a toluene (100 mL) solution of the pyrazole-4-carbonitrile of
12 Example 5 (3.5 g, 4.4 mmole) was added diethoxymethyl acetate (1.1
13 mL, 6.7 mmole). The solution was kept at 80-90°C for 5 hours and then
14 evaporated to a syrup. The syrup was dissolved in dichloromethane (10
15 mL) and added to ice cold methanolic ammonia (100 mL) in a glass
16 pressure bottle. After two days at RT the contents of the bottle were
17 evaporated to dryness. The residue was dissolved in methanol and
18 adjusted to pH 8 with freshly prepared sodium methoxide to complete
19 the deprotection. After stirring overnight the solution was treated with
20 Dowex^R-50 H+ resin, filtered and evaporated to dryness. The residue
21 was chromatographed on silica gel using acetone/hexane (3/2) as eluent
22 to give 2.0 g (77%) of analytically pure product.

23 EXAMPLE 7:

24 1-(2-Deoxy- β -D-erythropentofuranosyl)-3-[5-(trityl-
25 amino)-pentyl]pyrazolo[3,4-d]pyrimidin-4-amine 5'-monophosphate.

26 To an ice cold solution of the pyrazolopyrimidin-4-amine of
27 Example 6 (250 mg, 0.43 mmole) in trimethyl phosphate (5 mL) was
28 added phosphoryl chloride (50 μ L) and the solution was kept at 0-4°C.
29 The reaction was monitored by reversed phase HPLC using a linear
30 gradient from 0 to 100% acetonitrile in water over 25 min. After
31 stirring for 5 hours, an additional aliquot of phosphoryl chloride (25 μ L)

1 was added and the solution was stirred another 30 min. The solution
2 was poured into 0.1M ammonium bicarbonate and kept in the cold
3 overnight. The solution was then extracted with ether and the aqueous
4 layer evaporated to dryness. The residue was dissolved in water (5 mL)
5 and purified by reversed phase HPLC using a 22mm X 50cm C18
6 column. The column was equilibrated in water and eluted with a
7 gradient of 0 to 100% acetonitrile over 20 min. Fractions containing
8 the desired material were pooled and lyophilized to give 160 mg (56%)
9 of chromatographically pure nucleotide. EXAMPLE 8:

10 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-{5-[(6-bio-
11 tinamido)hexanamido]pentyl}pyrazolo[3,4-d]pyrimidin-4- amine
12 5'-monophosphate.

13 An ethanol solution (10 mL) of the nucleotide of Example 7,
14 palladium hydroxide on carbon (50 mg), and cyclohexadiene (1 mL) was
15 refluxed for 3 days, filtered, and evaporated to dryness. The residue
16 was washed with dichloromethane, dissolved in DMF (1.5 mL)
17 containing triethylamine (100 mL), and treated with
18 N-hydroxy-succinimidyl biotinylaminocaproate (50 mg). After stirring
19 overnight an additional amount of N-hydroxysuccinimidyl
20 6-biotinamidocaproate (50 mg) was added and the solution was stirred
21 for 18 hours. The reaction mixture was evaporated to dryness and
22 chromatographed following the procedure in Example 7. Fractions were
23 pooled and lyophilized to give 80 mg of chromatographically pure
24 biotinamido-substituted nucleotide.

25 EXAMPLE 9:

26 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(6-biotin-
27 amido)-hexanamidopentyl]pyrazolo[3,4-d]pyrimidin-4-amine
28 5'-triphosphate.

29 The monophosphate of Example 8 (80 mg, ca. 0.1 mmole) was
30 dissolved in DMF with the addition of triethylamine (14 μL).
31 Carbonyldiimidazole (81 mg, 0.5 mmole) was added and the solution

1 stirred at RT for 18 hours. The solution was treated with methanol (40
2 μ L), and after stirring for 30 minutes tributylammonium pyrophosphate
3 (0.5 g in 0.5 mL DMF) was added. After stirring for 24 hours another
4 aliquot of tributylammonium pyrophosphate was added and the solution
5 was stirred overnight. The reaction mixture was evaporated to dryness
6 and chromatographed following the procedure in Example 8. Two
7 products were collected and were each separately treated with conc.
8 ammonium hydroxide (1 mL) for 18 hours at 55°C. UV and HPLC
9 analysis indicated that both products were identical after ammonia
10 treatment and were pooled and lyophilized to give 35.2 mg of
11 nucleoside triphosphate.

12 EXAMPLE 10:

13 NICK-TRANSLATION REACTION

14 The triphosphate of Example 9 was incorporated into pHPV-16
15 using the nick translation protocol of Langer et al. (supra). The probe
16 prepared with the triphosphate of Example 9 was compared with probe
17 prepared using commercially available bio-11-dUTP (Sigma Chemical
18 Co). No significant differences could be observed in both a filter
19 hybridization and in in situ smears.

20 More specifically, the procedure involved the following materials
21 and steps

22 Materials:

23 DNase (ICN Biomedicals) - 4 μ g/mL

24 DNA polymerase 1 (U.S. Biochemicals) -
25 8 U/mL

26 pHPV - 16 - 2.16 mg/mL which is a
27 plasmid containing the genomic
28 sequence of human papillomavirus
29 type 16.

30 10X-DP - 1M Tris,pH7.5(20mL); 0.5M
31 OTT(80 mL); 1M MgCl₂(2.8 mL);

1 H₂O(17mL)
2 Nucleotides - Mix A - 2mM each dGTP,
3 dCTP, TTP (Pharmacia)
4 Mix U - 2mM each dGTP, DcTP,
5 dATP
6 Bio-11-dUTP - 1.0 mg/mL (BRL)
7 Bio-12-dAPPTP - 1.0 mg/mL

8 Steps:

9 To an ice cold mixture of 10X-DP (4 mL), pHV-16 (2 mL),
10 nucleotide mix A (6 mL), Bio-12-dAPPTP (2 mL), and H₂O (20 mL)
11 was added DNase (1 mL) and DNA polymerase 1 (2.4 mL). The
12 reaction mixture was incubated at 16°C for 1 hour. The procedure was
13 repeated using Bio-11-dUTP and nucleotide mix U in place of
14 Bio-12-dAPPTP (comprising the triphosphate of Example 9) and
15 nucleotide mix A.

16 Nucleic acid was isolated by ethanol precipitation and hybridized
17 to pHPV-16 slotted onto nitrocellulose. The hybridized biotinylated
18 probe was visualized by a streptavidin-alkaline phosphatase conjugate
19 with BCIP/NBT substrate. Probe prepared using either biotinylated
20 nucleotide gave identical signals. The probes were also tested in an in
21 situ format on cervical smears and showed no qualitative differences in
22 signal and background.

23 EXAMPLE 11:

24 5-Amino-3-[(5-tritylamino)pentyl]pyrazole-4-carbox- amide.

25 Following the procedure of Example 2, except that
26 cyanoacetamide is used instead of malononitrile,
27 5-(tritylamino)pentylhydroxymethylececyanoacetamide is prepared from
28 6-(tritylamino)caproic acid. This is then treated with diazomethane to
29 give the methoxy derivative, following the procedures of Example 3,
30 which is then reacted with hydrazine monohydrate, as in Example 4, to
31 give 5-amino-3-[(5-tritylamino)-

1 pentyl]pyrazole-4-carboxamide. EXAMPLE 12:
2 4-Hydroxy-6-methylthio-3-[(5-tritylamino)pentyl]pyra-
3 zolo-[3,4-d]pyrimidine.

4 The carboxamide from Example 11 is reacted with potassium
5 ethyl xanthate and ethanol at an elevated temperature to give the
6 potassium salt of 4-hydroxypyrazolo[3,4-d]pyrimidine-6-thiol. This salt is
7 then reacted with iodomethane to give
8 4-hydroxy-6-methylthio-3-[(5-tritylamino)pentyl]pyrazolo[3,4-d]pyrimidin
9 e.

10 EXAMPLE 13:

11 1-(2-Deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-
12 (tritylamino)pentyl]pyrazolo[3,4-d]pyrimidin-6-amine.

13 Following the procedure of Example 5, the pyrazolopyrimidine of
14 Example 12 is treated with sodium hydride and reacted with
15 1-chloro-1,2-dideoxy-3,5-di-O-toluoylribofuranose. The resulting
16 compound is reacted with MCPBA and with methanolic ammonia, and
17 the toluoyl protecting groups are removed to give the product.

18 EXAMPLE 14:

19 1-(2-Deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-
20 (6-biotinamido)hexanamidopentyl]pyrazolo[3,4-d]pyrimidin- 6-amine
21 5'-monophosphate.

22 Following the procedure of Example 7, the pyrazolopyrimidine of
23 Example 13 is reacted with phosphoryl chloride to give the
24 corresponding 5'-monophosphate.

25 Following the procedure of Example 8, the above
26 5'-monophosphate is reacted with palladium/carbon and cyclohexadiene,
27 and the residue is reacted with N-hydroxy-succinimidyl
28 biotinylaminocaproate to give
29 1-(2-deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-
30 (6-biotinamido)hexanamidopentyl]pyrazolo[3,4-d]pyrimidin-6-amine
31 5'-monophosphate.

1 EXAMPLE 15:

2 1-(2-Deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-
3 (6-biotinamido)hexanamidopentyl]pyrazolo[3,4-d]pyrimidin-6-amine
4 5'-triphosphate.

5 Following the procedure of Example 9, the 5'-monophosphate of
6 Example 14 is treated with carbonyldiimidazole and then reacted with
7 tributylammonium pyrophosphate to give the corresponding
8 5'-triphosphate.

9 EXAMPLE 16:

10 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(trityl-
11 amino)-pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoylamine.

12 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(tri-
13 tylamino)pentyl]pyrazolo[3,4-d]pyrimidine-4-amine from Example 6 is
14 reacted with benzoyl chloride and pyridine to give
15 1-(2-deoxy-3,5-di-O-benzoyl-β-D-erythropentofuranosyl)-3-[5-(tritylamino
16)pentyl]pyrazolo- [3,4-d]-pyrimidine-4-dibenzoylamine. This is treated
17 with aqueous sodium hydroxide to partially deprotect the compound
18 giving
19 1-(2-deoxy-β-D-erythropentofuranosyl)-3-[5-(tritylamino)pentyl]pyra-
20 zolo[3,4-d]pyrimidine-4-benzoylamine.

21 EXAMPLE 17:

22 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(trifluoro-
23 acetamido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoyl- amine.

24 Following the procedure of Example 8, the benzoylamine of
25 Example 16 is treated with palladium hydroxide on carbon and then
26 with trifluoroacetic anhydride to give 1-(2-deoxy-β-D-erythropentofuran-
27 osyl)-3-[5-(trifluoroacetamido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoyl
28 amine.

29 EXAMPLE 18:

30 1-(2-Deoxy-5-O-dimethoxytrityl-β-D-erythropentofuran-
31 osyl)-3-[5-(trifluoroacetamido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoyl

1 amine 3'-O-(N,N-diisopropyl)phosphoramidite cyanoethyl ester.

2 The compound of Example 17 is reacted with dimethoxytrityl
3 chloride and pyridine to give the corresponding 5'-dimethoxytrityl
4 compound. This compound is then reacted with cyanoethyl
5 chloro-N,N-diisopropylphosphoramidite (according to the method of
6 Sinha et al., Nucleic Acids Res., 12:4539 (1984)) to give the
7 3'-O-activated nucleoside.

8 EXAMPLE 19

9 5-(4-Phthalimidobut-1-yn-1-yl)-2'-deoxyuridine

10 5-Iodo-2'-deoxyuridine (354 mg, 1 mmol) was dissolved in 10 mL
11 of dimethylformamide. Cuprous iodide (76 mg, 0.4 mmol),
12 tetrakis(triphenylphosphine)palladium(0) (230 mg, 0.2 mmol), and
13 triethylamine (200 mg, 2.0 mmol) were added. 4-Phthalimidobut-1-yne
14 (300 mg, 1.5 mmol) was added all at once and the reaction kept at 60°C
15 for three hours. The clear yellow reaction was then evaporated and
16 methylene chloride was added. Scratching of the flask induced
17 crystallization of nearly all of the product which was filtered and
18 recrystallized from 95% ethanol to give 335 mg (78%) of title
19 compound as fine, feathery needles.

20 EXAMPLE 20

21 5-(4-Phthalimidobut-1-yl)-2'-deoxyuridine

22 1.00 Gram of 5-(4-Phthalimidobut-1-yn-1-yl)-2'-deoxyuridine was
23 dissolved in 95% EtOH and about 3 g of neutral Raney nickel was
24 added. After 48 hours, the catalyst was removed by cautious filtration
25 and the filtrate was evaporated to a solid which was recrystallized from
26 methanol-water to give 960 mg (97%) of the title compound.

27 EXAMPLE 21:

28 5-(3-Iodoacetamidopropyl)-2'-deoxyuridine.

29 5-(3-Trifluoroacetamidoprop-1-yl)-2'-deoxyuridine (0.3 mmol) is
30 treated with ammonia and then with N-hydroxy-succinimidyl
31 α -iodoacetate (0.5 mmol). The reaction mixture is evaporated to

1 dryness and purified by chromatography to give
2 5-(3-iodoacetamidopropyl)-2'-deoxyuridine.

3 EXAMPLE 22

4 5-(4-(4-Bromobutyramido)butyl)-2'-deoxyuridine

5 5-(4-phthalimidobut-1-yl)-2'-deoxyuridine is treated with ammonia
6 and then with N-hydroxysuccinimidyl-4-bromobutyrate to give
7 5-(4-(4-bromobutyramido)butyl)-2'-deoxyuridine.

8 Preparation of Synthetic Oligonucleotides

9 EXAMPLE 23:

10 Phosphoramidite Preparation and DNA Synthesis.

11 Nucleosides were 5'-dimethoxytritylated, following known
12 procedures, to give around 85% yield, and the 3'-phosphoramidite was
13 made using diisopropylamino β -cyanoethylchlorophosphite (as described
14 in "Oligonucleotide Synthesis: A Practical Approach", supra) with
15 diisopropylethylamine in methylene chloride. The phosphoramidite was
16 made into a 0.2N solution in acetonitrile and placed on the automated
17 DNA synthesizer. Incorporation of these new and modified
18 phosphoramidites gave incorporation similar to ordinary
19 phosphoramidites (97-99% as judged by assay of the trityl color released
20 by UV.) Oligonucleotides were removed from the DNA synthesizer
21 in tritylated form and deblocked using 30% ammonia at 55°C for 6
22 hours. Ten μ L of 0.5M sodium bicarbonate was added to prevent
23 acidification during concentration. The oligonucleotide was evaporated
24 to dryness under vacuum and redissolved in 1.0 mL water. The
25 oligonucleotides were purified by HPLC using 15-55% acetonitrile in
26 0.1N triethylammonium acetate over 20 minutes. Unsubstituted
27 oligonucleotides came off at 10 minutes; amino derivatives took 11-12
28 minutes. The desired oligonucleotide was collected and evaporated to
29 dryness, then it was redissolved in 80% aqueous acetic acid for 90
30 minutes to remove the trityl group. Desalting was accomplished with a
31 G25 Sephadex column and appropriate fractions were taken. The

1 fractions were concentrated, brought to a specific volume, dilution
2 reading taken to ascertain overall yield and an analytical HPLC done to
3 assure purity. Oligonucleotides were frozen at -20°C until use.

4 Following the above procedures, the nucleoside
5 5-(3-trifluoroacetamidoprop-1-yl)-2'-deoxyuridine was converted to the
6 5'-O-dimethoxytrityl-3'-(N,N-diisopropyl)-phosphoramidite cyanoethyl
7 ester derivative. This was added to a DNA synthesizer and the
8 following 14-mer oligonucleotide sequence was prepared:

9 3'-CT TCC U¹TG TAG GTC-5'

10 where U¹ is 5-(3-aminoprop-1-yl)-2'-deoxyuridine (oligo A).

11 In the same manner, 5-(4-phthalimidobut-1-yl)-2'-deoxyuridine
12 was converted to the
13 5'-(O-dimethoxytrityl-3'-(N,N-diisopropyl)phosphoramidite cyanoethyl
14 ester derivative and added to a DNA synthesizer to prepare the above
15 14-mer oligonucleotide sequence where U¹ is
16 5-(4-aminobut-1-yl)-2'-deoxyuridine (oligo C).

17 A corresponding 14-mer oligonucleotide was also prepared where
18 U¹ is the unmodified deoxyuridine.

19 EXAMPLE 24:

20 Derivatization of Oligonucleotides.

21 In general, to add the crosslinking arm to an
22 aminoalkyloligonucleotide, a solution of 10 µg of the
23 aminoalkyloligonucleotide and a 100X molar excess of
24 n-hydroxysuccinimide haloacylate such as α-haloacetate or
25 4-halobutyrate in 10 µL of 0.1 M borate buffer, pH 8.5, was incubated
26 at ambient temperature for 30 min. in the dark. The entire reaction
27 was passed over a NAP-10 column equilibrated with and eluted with
28 distilled water. Appropriate fractions based on UV absorbance were
29 combined and the concentration was determined spectrophotometrically.

30 Introduction of the haloacyl moiety was examined by HPLC. A
31 Zorbax^R oligonucleotide column (Dupont) eluted with a 20 minute

1 gradient of 60% to 80% B composed of: A (20% acetonitrile:80% 0.02
2 N NaH_2PO_4) and B (1.2 N NaCl in 20% acetonitrile:80% 0.02 N
3 NaH_2PO_4). The presence of a reactive α -haloacyl moiety was indicated
4 by return of the retention time of the α -haloacylamidoalkyl
5 oligonucleotide to the corresponding aminoalkyl oligonucleotide after
6 exposure to 1N cysteamine. Introduction of cysteamine created
7 equivalent charge patterns between the aminoalkyl oligonucleotide and
8 the α -haloacylamido oligonucleotide.

9 Following the above procedure, the 14-mer oligonucleotide:

10 3'-CT TCC U¹TG TAG GTC-5'

11 where U¹ is 5-(3-aminoprop-1-yl)-2'-deoxyuridine (oligo A, Example 23),
12 was reacted with n-hydroxysuccinimide α -iodoacetate to give the above
13 14-mer oligonucleotide where U¹ is
14 5-(3-iodoacetamidoprop-1-yl)-2'-deoxyuridine (oligo B).

15 Oligo A and oligo B, as well as the above 14-mer where U¹ is the
16 unmodified deoxyuridine were resolved in the Zorbax column, all of
17 identical sequence, with the following retention times: unmodified
18 14-mer, 9.31 min; aminopropyl 14-mer (oligo A), 7.36 min; and
19 iodoacetamido-propyl 14-mer (oligo B), 10.09 min.

20 In the same manner, the aminopropyl 14-mer (oligo A) was
21 reacted with N-hydroxysuccinimide 4-bromobutyrate to give the 14-mer
22 where U¹ is 5-(3-(4-bromobutyramido)prop-1-yl)-2'-deoxyuridine.

23 Also, the aminobutyl 14-mer (oligo C, Example 23) was reacted
24 with either N-hydroxysuccinimide α -iodoacetate or
25 N-hydroxysuccinimide 4-bromobutyrate to give the 14-mer where U¹ is
26 5-(4-iodoacetamidobut-1-yl)-2'-deoxyuridine or
27 5-(4-(4-bromobutyramido)but-1-yl)-2'-deoxyuridine, respectively.

28 Assays

29 EXAMPLE 25:

30 Assay of Crosslinking Reaction to Single Stranded DNA

31 The reaction of crosslinking a DNA probe to a target nucleic acid

1 sequence contained 1 μ g of haloacylamidoalkyl probe and 10 ng of
 2 32 P-labeled cordycepin-tailed target in 200 μ L of 0.1 M Tris, pH 8.0, and
 3 0.9 M NaCl incubated at 20° or 30°C. Aliquots were removed at 24- or
 4 72-hour intervals and diluted in 20 μ L of 10 mM cysteamine to quench
 5 the haloacylamido group. These solutions were stored at RT, and 1 μ L
 6 was used for analysis by denaturing polyacrylamide gel electrophoresis
 7 (PAGE).

8 Following the above procedure, two model oligonucleotide
 9 sequences were utilized to evaluate the crosslinkage potential of the
 10 modified probe to its complement. The sequences, derived from human
 11 papillomavirus (HPV) or human cytomegalovirus (CMV), are shown
 12 below:

13
 14 HPV System:

15 5 10 15 20 25 30

16
 17 Target: 5'-AGA CAG CAC AGA ATT CGA AGG AAC ATC CAG-3'
 18 Probe: 3'-CT TCC UTG TAG GTC-5'

19
 20
 21 CMV System:

22
 23 5 10 15 20

24
 25 Target: 5'-ACC GTC CTT GAC ACG ATG GAC TCC-3'
 26 Probe: 3'-GAA CTG TGC UAC CTC-5'

27
 28 U = 5-[3-(α -iodoacetamido)- or 3-(4-bromobutyryl-
 29 amido)-propyl]-2'-deoxyuridine, or

30 U = 5-[3-(α -iodoacetamido)- or 4-(4-bromobutyryl-
 31 amido)-butyl]-2'-deoxyuridine.

32 The target for HPV is a 30-mer, and for CMV it is a 24-mer.

33 The crosslinking probes were a 14-mer for HPV and two 15-mers for

34 CMV. Each probe contained a single modified deoxyuridine designated

1 as U in the sequences above.

2 The reaction of HPV target with a limiting amount of crosslinking
3 probe containing a 5-(3-iodoacetamidopropyl) sidearm can be analyzed
4 in a cleavage pattern on a denaturing PAGE gel, and the analysis
5 showed the loss of the crosslinked hybrid with the concomitant
6 appearance of a discrete low molecular weight band. The intensity of
7 this band was dependent upon the extent of crosslinkage in the initial
8 reaction. The localization of signal into two discrete bands on the gel
9 strongly argues that no non-sequence-directed alkylation of either target
10 or probe strands had occurred (including intramolecular probe
11 alkylation).

12 Comparison with an authentic 15-mer run in an adjacent lane
13 suggested that the major cleaved fragment is a 9-mer. Upon close
14 examination of the original autoradiogram, a slower moving band of
15 very weak intensity was visible. This pattern would be consistent with
16 major alkylation at G-21 and minor alkylation at G-20. An examination
17 of a Dreiding model of the crosslinkable HPV hybrid shows that the
18 5-(3-iodoacetamidopropyl) sidearm can contact the G-21 residue of the
19 target strand with only minor distortion of the helix.

20 If alkylation occurs predominately at a guanosine on the target
21 strand located two units on the 5' side of the modified-deoxyuridine
22 base pair, the CMV sequence should not react. This result was in fact
23 observed. The absence of reaction with CMV further supports the
24 specificity of the crosslinking scheme of the invention.

25 EXAMPLE 26:

1 Time and Temperature Dependence.

2 Time and temperature dependence studies were carried out with
3 the HPV system of Example 25 where U is
4 5-(3-iodoacetamidoprop-1-yl)-2'-deoxyuridine. The target was
5 ³²P-labeled by cordycepin tailing with terminal transferase (Maniatis et
6 al., "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor
7 Laboratory, 1982, p. 239) and incubated with excess probe in a pH 8.0
8 Tris buffer at either 20° or 30°C. Aliquots were removed after 0, 24, or
9 72 hours incubation, quenched with an equivalent volume of 10 mM
10 mercaptoethylamine (which reacts with the iodoacetamide), and stored
11 at RT for subsequent analysis by denaturing or non-denaturing PAGE.

12 Crosslinkage of the hybrid, which was monitored by denaturing
13 PAGE, was evident for the 24 and 72 hour time points at both
14 temperatures. The amount of crosslinked hybrid increased with both
15 temperature and time. Approximately 20% of the hybrid was
16 crosslinked after 72 hours incubation at 30°C.

17 Separate experiments at a range of temperatures indicated that
18 the half-life for crosslinking at 37°C is approximately 2 days, and that
19 the reaction is complete after 24 hours at 58°C. This time-dependent
20 reaction implies that the iodoacetamido moiety does not hydrolyze or
21 react with the buffer. The increased reaction rate at higher temperature
22 indicates that the hybrid is maintained, and subsequently the rate of
23 alkylation shows the expected increase with temperature.

24 EXAMPLE 27:

25 Site Specificity of Alkylation.

1 to elucidate the site specificity of alkylation, the crosslinked HPV
2 hybrid of Example 25 (where U is
3 5-(3-iodoacetamidoprop-1-yl)-2'-deoxyuridine) was subjected to a 10%
4 piperidine solution at 90°C for 60 minutes. As shown by Maxam et al.
5 (Proc. Natl. Acad. Sci. USA 74:560 (1977)), this treatment quantitatively
6 cleaves the target strand 3'-to the site of alkylation. The resulting data
7 indicated that the alkylation of the second guanine above the
8 crosslinker-modified base pair (i.e., the guanine above the target base)
9 was the exclusive reaction observed, indicating that the crosslinking
10 reaction in the HPV model system is remarkably specific.

11 CROSS-LINKING OLIGONUCLEOTIDES HAVING TWO
12 CROSS-LINKING FUNCTIONS FOR ANTI-GENE THERAPY AND
13 APPLICATION AS PROBES FOR DOUBLE STRANDED DNA AND
14 DNA MAPPING

15 In accordance with the second broad aspect of the invention the
16 ODN has at least two covalently attached cross-linking agents. The
17 ODN in this aspect of the invention preferably has no more than
18 approximately 300, and preferably no more than approximately 60
19 nucleotide units. The cross linking agents may be attached at the 3' or
20 5' phosphate terminus, or to the sugar or any heterocyclic base within
21 the ODN. A cross-linking agent which has two cross-linking
22 functionalities, such as a crosslinking agent having the formula
23 -N-[(CH₂)₂-L]₂ (a bifunctional N-mustard) is capable of two alkylations,
24 and is therefore considered as two cross-linking agents in this aspect of
25 the invention. The ODN bearing the two cross-linking agents in

1 accordance with this aspect of the invention is complementary in the
2 Hoogsteen or reverse Hoogsteen pairing sense to the target double
3 stranded DNA. There is evidence in accordance with this aspect of the
4 invention that when the ODN has one covalently attached bifunctional
5 cross linking agent (bifunctional N-mustard) then after triplex formation
6 the modified ODN attaches to both strands of the target double
7 stranded DNA sequence. Alternatively, cross-linking agents are
8 attached to at least two different sites of the ODN. In this case also,
9 evidence indicates that two separate covalent bonds are formed with the
10 target DNA sequence. In both situations, in accordance with this aspect
11 of the invention, formation of at least two covalent bonds with the
12 target DNA is attained.

13 As noted above, the modified ODN of the present invention
14 forms a triplex with the target double stranded DNA sequence. This is
15 followed by cross-linkage which inactivates the target DNA sequence.
16 Following triple strand formation and covalent crosslinkage, the
17 modified target DNA no longer supports replication or transcription.
18 Unlike all other lesions in DNA, however, this modification is much less
19 likely to be repaired by the organism. Normally, cross-linked DNA is
20 repaired by a combination of excision repair and homologous
21 recombination. With cross-linked triple strand complexes, however,
22 there will be no undamaged copies of the targeted gene to participate in
23 recombination. By analogy with procaryotic models, the eucaryotic cell
24 may attempt to use a misrepair (or SOS) pathway wherein the crosslink
25 will be removed, but at the expense of mutagenesis. In such case, gene

1 function is likely to be irreversibly silenced by the resultant mutations.

2 The use of recombination enzymes in combination with
3 anti-gene ODNs significantly enhances the efficiency with which the
4 single strand ODN "finds" its complementary target DNA sequence.
5 Accordingly, the efficiency of triple strand formation is greatly increased
6 when the anti-gene ODN is combined with a recombination enzyme (for
7 instance, in a nucleoprotein complex).

8 Within the present invention, suitable target DNA
9 sequences include deleterious structural genes and both associated
10 up-stream and down-stream regulatory control sequences so that their
11 deactivation brings about a therapeutically beneficial result. Target
12 sequences also include genes of invading organisms, such as viral, fungi,
13 parasites, bacteria and tumor cells. The regulatory sequences may be
14 involved in either transcription or replication. The anti-gene ODN is
15 determined and designed according to the target DNA sequence chosen
16 for alteration of function, and has a sequence complementary in the
17 Hoogsteen or reverse Hoogsteen sense to a homologue run in one of
18 the two strands of the chosen target DNA.

19 In a particularly preferred embodiment, an antigenic ODN is
20 administered to a cell or a host, and upon entry to a target cell nucleus,
21 the anti-gene ODN combines with recombination enzymes present
22 within the nucleus. In this mode the ODN can bind to any
23 complementary sequence of choice. In this mode the ODN can bind to
24 any complementary sequence of choice. In an alternative embodiment,
25 the anti-gene ODN and recombination enzyme are combined ex vivo

1 and then administered to a cell or a host as a nucleoprotein filament.
2 In this embodiment, it may be advantageous to administer the
3 nucleoprotein filament in a liposome. Preferred recombination enzymes
4 include procaryotic and eucaryotic recombination enzymes, such as
5 recA, human recombinase and Drosophila recombinase, with human
6 recombinase particularly preferred.

7 As it was noted above, experimental evidence proves that both of
8 the minimum two cross-linking agents incorporated into the modified
9 ODNs of the present invention actually react with the target DNA
10 sequence, and that both strands of the target sequence are subsequently
11 covalently linked and should be deactivated by the modified ODN.

12 Another important use or application of the modified ODNs of
13 the present invention is in the field of mapping large DNA molecules,
14 and related analytical and investigative techniques in the fields of
15 molecular biology, genetics and biochemistry. For example, the ability
16 to chemically restrict or cleave long double-stranded DNA at unique
17 sites 10-20 base pairs in length has been frequently discussed as a
18 technique which could accelerate the human genome project by
19 providing a means for physically mapping large DNA molecules. In
20 accordance with this aspect of the present invention a triple-stranded
21 complex is allowed to form between a synthetic oligonucleotide (ODN)
22 and a "complementary" 10-20 base long homopurine run in
23 double-stranded DNA. Since the modified ODN of the present
24 invention is appropriately appended with two alkylating agents,
25 crosslinkage to two guanine residues on opposite strands of the duplex

1 takes place. Crosslinks to guanine residues are known in the art to
2 render the alkylated DNA susceptible to cleavage, for example through
3 cleavage of the glycosidic bond between the alkylated guanine base and
4 the sugar moiety, followed by cleavage of the phosphodiester bond.
5 The overall cleavage of the alkylated DNA at the alkylation sites may
6 occur spontaneously, or as a result of an appropriate enzyme or other
7 reagent acting on the modified DNA. In accordance with the presently
8 preferred mode of this aspect of the invention the alkylated duplex
9 DNA is incubated with an amino acid, lysine, arginine or histidine, or
10 with a DNA associating protein (such as a histone or a recombinase
11 enzyme). This converts each alkylation site into a cleavage site probably
12 through the process of depurination (cleavage of glycosidic bond of the
13 alkylated guanine residue) followed by beta elimination reaction.

14 More specifically, still in connection with the use of the modified
15 ODNs of the present invention for "DNA mapping" or "gene mapping"
16 or related investigative procedures, the following is noted. The structure
17 of the modified ODN used in the process is known in accordance with
18 the present invention. Thus, the modified ODN can be tailor made to
19 cleave at one or more specific approximately 10 to 20 base pair regions
20 (target regions) of the target DNA. The structure of the target region
21 may be known already, in which case the modified ODN is created
22 specifically for the target region pursuant to the rules of Hoogsteen or
23 reverse Hoogsteen pairing. Alternatively, no specific site in the target
24 DNA may be known, in which case the site of cleavage brought about
25 as a result of hybridization with a modified ODN of known sequence

1 nevertheless provides information about the existence and number of
2 "matching" regions in the target DNA.

3 Thus, in accordance with this aspect of the invention and
4 examples, a double-stranded plasmid DNA which contains a 20 base
5 pair long homopurine/homopyrimidine run is chemically restricted. To
6 effect cleavage 1-10 μ g of the plasmid is incubated with 1-10 μ M of a
7 C⁺/T, G/A or G/T motif 20 mer ODN designed to form a sequence
8 specific triple strand with the homopurine run using the Hoogsteen or
9 reverse Hoogsteen base pairing rules. (For an A rich homopurine run a
10 C⁺/T or G/A motif ODN is employed; for a G rich homopurine run a
11 G/A or G/T motif ODN is used.) Triplexing is carried out overnight at
12 15-37° C in the presence of 10 mM MgCl₂ at pH 6.0 (C⁺/T motif) or pH
13 7.0-7.5 (G/A or G/T motif). The C⁺ symbol in the C⁺/T motif stands
14 for the 5-methylcytosine base which is better suited for Hoogsteen or
15 reverse Hoogsteen pairing than cytosine.

16 The modified ODN which becomes the third strand during the
17 incubation may have the two alkylating groups appended to either
18 internal base residues or to the 5' or 3' terminus. Each alkylating group
19 reacts with the N-7 position of a nearby guanine residue of the targeted
20 duplex. Double stranded break of the duplex occurs because the two
21 guanine residues reside on opposite strands of the duplex. In the case
22 where the alkylating group is attached to an internal base on the third
23 strand ODN, that base is purposely designed to form a mismatch with
24 the opposing G-C or C-G base pair in the targeted duplex. This allows
25 access to the N-7 position of guanine by the alkylator. By contrast,

1 when the alkylating group is attached to the end of the ODN so as to
2 target a guanine residue in the flanking duplex, the terminal base in the
3 ODN is designed to hydrogen bond to the opposing base pair of the
4 target. The general rules for placement of alkylating groups on the
5 ODNs are illustrated by the three examples below where the upper
6 strand is the crosslinkable third strand ODN; X represents a terminal
7 alkylating group while Y represents an alkylating group attached to an
8 internal 5-(3-aminopropyl)-2'-deoxyuridine residue. The guanine bases
9 crosslinked in the target duplex are bold faced and underlined. The
10 crosslinkable ODN is identified by a Sequence No. and the double
11 stranded target, is also identified by a single Sequence No.

12 Example 1 G/A motif ODN targeted to the homopurine run in human
13 HLA DQB1 0302 allele:

14 3'-XGAGAGAGGAAAGAGGAGAX Sequence No.

15 1

16 5'-ATATAAGGAGAGAGGAAAGAGGAGACAAA

17 3'-TATATTCCTCTCTCCTTTCTCCTCTGTTT Sequence No. 2

18 Example 2 G/T motif ODN targeted to the homopurine run in human
19 epidermal growth factor receptor:

20 3'-GGGTGGTGYTGTGYTGGTGGTGTT Sequence No.

21 3

22 5'-GGGAGGAGCAGAGGAGGAGGAGAA

23 3'-CCCTCCTCGTCTCCTCCTCCTCTT Sequence No. 4

24 Example 3 C⁺/T/G motif ODN targeted to a homopurine run in HIV
25 proviral DNA:

1 5'-TTTTCTTTYGGGGGTX

Sequence No. 5

2 5'-TTTTTAAAAGAAAAGGGGGGACTGG

3 3'-AAAAATTTTCTTTTCCCCCCTGACC

Sequence No. 6

4 After complexing the modified ODN to the target duplex and
5 cross-linking the crosslinked DNA is incubated 12-24 hr at 37° with 10
6 mM lysine, arginine or histidine. As noted above, as a result of this
7 procedure each crosslink is converted into a nick through a depurination
8 and beta-elimination pathway. If the alkylated guanines are within 5-6
9 base pairs of one another the staggered nicks break the DNA;
10 otherwise, breakage is be accomplished by brief incubation with
11 exonuclease III (3' to 5' digestion) or calf spleen phosphodiesterase (5'
12 to 3' digestion) to remove a portion of the intervening duplex. The
13 choice of exonuclease depends upon the polarity of the target strands in
14 relation to the positions of the nicks. If necessary, prior to exonuclease
15 treatment the triplex can be destroyed by complexing magnesium ion
16 with excess EDTA and then spinning the sample through a disposable
17 gel filtration cartridge. The spin simultaneously removes the ODN and
18 exchanges the plasmid into exonuclease buffer. After digestion the
19 samples can be phenol extracted and alcohol precipitated for use in
20 later experiments.

21 Specific Embodiments and Experimental

22 Procedures 2,3,5,6-Tetrafluorophenyl trifluoroacetate.

23 A mixture of 2,3,5,6-tetrafluorophenol (55.2 g, 0.33 mol),
24 trifluoroacetic anhydride (60 mL, 0.42 mol) and boron trifluoride
25 etherate (0.5 mL) was refluxed for 16 hr. Trifluoroacetic anhydride and

1 trifluoroacetic acid were removed by distillation at atmospheric
2 pressure. The trifluoroacetic anhydride fraction (bp 40°C) was returned
3 to the reaction mixture along with 0.5 mL of boron trifluoride etherate,
4 and the mixture was refluxed for 24 hr. This process was repeated two
5 times to ensure complete reaction. After distillation at atmospheric
6 pressure, the desired product was collected at 62°C/45 mm (45°C/18
7 mm) as a colorless liquid: yield = 81.3 g (93%); d = 1.52 g/mL; n_D^{21} =
8 1.3747; IR (CHCl_3) 3010, 1815, 1525, 1485, 1235, 1180, 1110, and 955
9 cm^{-1} . Anal. Calcd for $\text{C}_8\text{HF}_7\text{O}_2$: C, 36.66; H, 0.38; F, 50.74. Found: C,
10 36.31; H, 0.43; F, 50.95.

11 2,3,5,6-Tetrafluorophenyl-4'-[bis(2-chloroethyl)amino]phenylbutyrate
12 (Chlorambucil 2,3,5,6-tetrafluorophenyl ester)

13 To a solution of 0.25 g (0.82 mmol) of chlorambucil (supplied by
14 Fluka A. G.) and 0.3 g (1.1 mmol) of 2,3,5,6-tetrafluorophenyl
15 trifluoroacetate in 5 ml of dry dichloromethane was added 0.2 ml of dry
16 triethylamine. The mixture was stirred under argon at room temperature
17 for 0.5 h and evaporated. The residual oil was purified by column
18 chromatography on silica gel with hexane-chloroform (2:1) as the eluting
19 solvent to give the ester as an oil: 0.28 g (75%); TLC on silica gel
20 (CHCl_3) R_f 0.6; IR (in CHCl_3) 3010, 1780, 1613, 1521, 1485 cm^{-1} .

21 2-Propargyloxyethyl)amine (John, R., and Seitz, G., Chem. Ber., **123**,
22 133 (1990) was prepared by condensing propynol with
23 2-bromoethylammonium bromide in liquid ammonia in the presence of
24 Na NH_2 , and was used crude for the next reaction.

25 3-(2-Trifluoroacetamidoethoxy)propyne

1 (2-Propargyloxyethyl)amine (13.8 g, 0.14 mol) is stirred and
2 chilled in an iso-propanol-dry ice bath while excess of trifluoroacetic
3 anhydride (26 ml, 0.18 mol) is added dropwise.
4 N-(2-Propargyloxyethyl)trifluoroacetamide is distilled at 84-85°/1.7 torr
5 as an oil which solidified upon refrigeration; yield 14.4 g (52%), m.p.
6 (16°, n_D^{24} 1.4110. Anal. Calcd. for $C_7H_8F_3NO_2$: C, 43.09, H, 4.13; N, 7.18;
7 F, 29.21. Found: C, 42.80; H, 4.03; N, 7.06; F, 29.38.

8 5-[3-(2-Trifluoroacetamidoethoxy)propynyl]-2'-deoxyuridine

9 A mixture of 5-iodo-2'-deoxyuridine (3.54 g, 10 mmol), copper(I)
10 iodide (0.19 g, 1 mmol) and tetrakis(triphenylphosphine)palladium(O)
11 (0.58 g, 0.5 mmol) is dried in vacuo at 60° for 3 hours and placed under
12 argon. A suspension of the mixture in dry DMF (20 ml) is stirred under
13 argon and treated with dry triethylamine (1.7 ml, 12 mmol) followed by
14 3-(2-Trifluoroacetamidoethoxy)propyne (3.17 g, 16 mmol). The mixture
15 is cooled at room temperature in a water bath and stirred for 17 hours.
16 The mixture is treated with 2% acetic acid (100 ml), the catalyst is
17 removed by filtration and washed with 50% methanol. The filtrates are
18 combined and passed onto a LiChroprep RP-18 column (5X25 cm), the
19 column is washed, then eluted with 1% acetic acid in 50% (v/v)
20 methanol. The fractions with the main product are combined,
21 evaporated, and dried in vacuo. The resultant foam is stirred with 150
22 ml of ether to give crystalline product; yield 3.6 g (85%); m.p. 145-152°.

23 5-[3-(2-Trifluoroacetamidoethoxy)propyl]-2'-deoxyuridine

24 A solution of 5-[3-(2-trifluoroacetamidoethoxy)-
25 propynyl]-2'-deoxyuridine (3.4 g, 8.1 mmol) in methanol (20 ml) is

1 stirred with ammonium formate (prepared by addition of 3 ml, 79 mmol
2 of cold 98% formic acid into 2 ml, 50 mmol of dry ice frozen 25%
3 ammonia) and 0.2 g of 10% Pd/C for 7 hours at room temperature
4 under hydrogen atmosphere. The catalyst is removed by filtration, the
5 filtrate evaporated and product is purified on LiChroprep RP-18 column
6 by the above procedure. Fractions containing the desired product are
7 combined and evaporated to dryness in vacuo and the resultant solid is
8 triturated with dry ether to give 3.0 g (87% product, m.p. 107-110°; λ_{\max}
9 in nm, in 0.1M triethylamine-acetate (pH 7.5), 220, 268. Analysis
10 calculated for $C_{16}H_{22}F_3N_3O_7$: C, 45.18; H, 5.21; N, 9.88; F, 13.40. Found
11 C, 45.16; H, 5.16; N, 9.68; F, 13.13.

12 Preparation of Synthetic Oligonucleotides

13 Introduction of chlorambucil residue into the primary amino groups of 14 oligonucleotides

15 Preparation of the cetyltrimethylammonium salt of oligonucleotides: a
16 100 μ L aliquot of aqueous solution of oligonucleotide (50-500 ug),
17 generally triethylammonium salt, was injected to a column packed with
18 Dowex 50wx8 in the cetyltrimethylammonium form and prewashed with
19 50% alcohol in water. The column was eluted by 50% aqueous ethanol
20 (0.1 mL/min). Oligonucleotide containing fraction was dried on a
21 Speedvac over 2 hours and used in following reactions.

22 Ethanol solution (50 μ L) of cetyltrimethylammonium salt of an
23 oligonucleotide (50-100 μ g) was mixed with 0.08 M solution of
24 2,3,5,6-tetrafluorophenyl-4'-[bis(2-chloroethyl)amino]phenylbutyrate
25 (tetrafluorophenyl ester of chlorambucil) in acetonitrile (50 μ L) and 3

1 μ L of diisopropylethylamine. After shaking for three hours at room
2 temperature, the product was precipitated by 2% LiClO₄ in acetone (1.5
3 mL). The product was reprecipitated from water (60 μ L) by 2% LiClO₄
4 in acetone three times. Finally the chlorambucil derivative of the
5 oligonucleotide was purified by Reverse Phase Chromatography with
6 approximately 50-80% yield. The fraction containing the product was
7 concentrated by addition of butanol. The isolated chlorambucil
8 derivative of the oligonucleotide was precipitated in acetone solution
9 with LiClO₄, washed by acetone and dried under vacuum. All
10 manipulations of reactive oligonucleotide were performed as quickly as
11 possible, with the product in ice-cold solution. Demonstration of
12 crosslinking of the third-strand oligonucleotide to both strands of a
13 duplex DNA target within a triplex

14 The following sequences were used to demonstrate bifunctional
15 crosslinking:

16 C: 5'-XCTTTCCTCTCTTTCCCCX-3' Seq. 7

17 A:

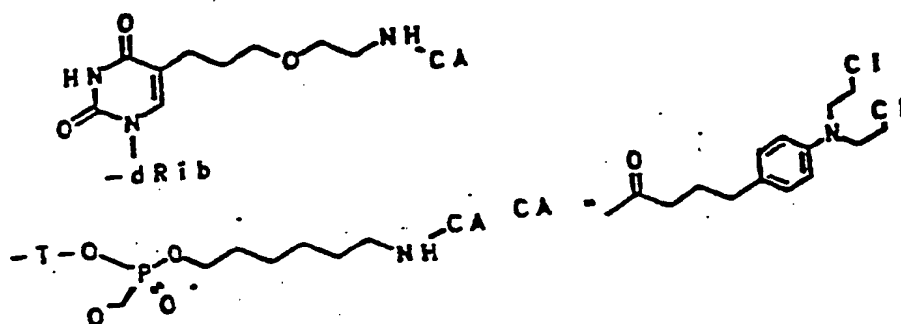
18 5'-AAATACTGGGAGAAAGGAGAGAAAAGGGGACCCAACGTAT
19 -3'

20 B: 3'-TTTATGACCCTCTTTCCTCTCTTTCCCCTGGGTTGCATA-5'
21 Seq.8

22 The strands A and B form the Watson-Crick duplex by
23 conventional base pairing, and C is the third strand, which pairs to
24 strand A within the duplex by Hoogsteen or reverse Hoogsteen
25 hydrogen bonding. The X residues in strand C bear the alkylating

1 moiety which is shown by the formulas below. In this example, the
2 p-[bis(2-chloroethyl)amino]phenylbutyrate group (designated CA) is
3 bound to, in one case, a 5-(aminoethoxypropyl)deoxyuridine, which is
4 the terminal nucleotide at either the 3'- or 5'-end, or at both ends, of
5 the oligonucleotide C. In the second case, the alkylating group (CA) is
6 bound to an aminohexyl group esterified to a phosphate at either the 3'-
7 or 5'-end, or to both ends, of the oligonucleotide. The alkylating
8 residue CA is placed onto the reactive amino group of the
9 oligonucleotide by the method described above.

10 Oligonucleotides were 5'- labeled with [γ - ^{32}P]ATP from DuPont (NEN
11 Research Products; Boston, MA) and T4 polynucleotide kinase from
12 United States Biochemical (Cleveland, OH) using the procedure of
13 Maxam and Gilbert (Maxam,A.M.;Gilbert,W. (1980) Methods in
14 Enzymology, 65, 499). The ^{32}P -labeled product was purified using a
15 Dupont NensorbTM 20 column (Wilmington, DE). Cerenkov counting
16 was done on a Beckman LS 5000TD from Beckman Instruments, Inc.
17 (Fullerton, CA). Oligonucleotide concentrations were calculated from
18 A_{260} values.



Each hybridization mixture contained 5 μ L of the labelled oligonucleotide in water, conc 5×10^{-7} M, was mixed with 10 μ L of complementary unlabelled strand, at the same conc, and with 5 μ L of a buffer which contained 350 mM NaCl, 100 mM $MgCl_2$, and 125 mM Na cacodylate, pH 6.0. After mixing, the mixture was incubated at 37° for 1 hr, and then 5 μ L of a solution of reactive oligonucleotide (which had been kept ice-cold until this time), conc 5×10^{-5} , was added, and incubation was continued at 37° for various times. Electrophoresis shows the formation of the slower moving bands that correspond to all three strands in the system being covalently linked together, indicating bifunctional crosslinking.

The position of cleavage was ascertained by treatment of the incubation mixtures with 1 M pyrrolidine in water for 15 min, 2x evaporation from water, and then polyacrylamide gel electrophoresis on a 20% gel. This analysis showed specific cleavage of the labeled target

1 strand at the expected site; specifically, the guanines immediately 5' to
2 the strand C binding site on both strands A and B, which are indicated
3 in the formula of the strands by underlining.

4 CROSS-LINKING OLIGONUCLEOTIDES HAVING A
5 CROSS-LINKING FUNCTION FOR ANTI-GENE THERAPY AND
6 APPLICATION AS PROBES FOR DOUBLE STRANDED DNA, WITH
7 A SEQUENCE HOMOLOGOUS TO ONE STRAND OF THE TARGET
8 DNA

9 In accordance with a third aspect of the present invention an
10 ODN has at least one covalently attached cross-linking function and at
11 least approximately 26 nucleotide units in a continuous sequence which
12 are homologous to a target sequence in one strand of double stranded
13 DNA (dsDNA). The ODN in this aspect of the invention has no more
14 than approximately 3000 nucleotide units, preferably no more than
15 approximately 300, and still more preferably no more than
16 approximately 60.

17 Broadly speaking the cross-linking function is of the structure
18 described above, namely it is an A-L or A-L₂ function, covalently
19 attached either to an internal nucleotide unit or to a terminal nucleotide
20 unit of the ODN. The nature of the A-L and A-L₂ function, the
21 meaning of the A and L symbols and specific embodiments of these
22 functions have been described above in connection with the first and
23 second main embodiments or aspects of the invention. Presently
24 preferred embodiments of the cross-linking function for crosslinking
25 with double stranded DNA in accordance with this aspect of the

1 invention include the α -haloacyl function as the reactive group, and the
2 N-mustard type reactive group. Even more preferably the N-mustard
3 type reactive group is attached to a 5-(3-aminopropyl) (or like)
4 substituted 2'-deoxyuridine unit of the ODN. The N-mustard type
5 reactive group is preferably a bis (2-chloroethyl)amine, more preferably
6 derived from chlorambucil, and therefore has the structure
7 $\text{CO}-(\text{CH}_2)_3-\text{C}_6\text{H}_4-\text{N}-(\text{CH}_2\text{CH}_2\text{Cl})_2$. As noted above this "chlorambucil"
8 moiety can be attached to the amino group of 5-(3-aminopropyl) (or
9 like) substituted 2'-deoxyuridine unit of the ODN by reacting the ODN
10 with
11 2,3,5,6-tetrafluorophenyl-4'-[bis(2-chloroethyl)amino]phenylbutyrate.
12 Alternatively, in another preferred embodiment the cross-linking
13 function is covalently attached to the 5' or 3'-end of the ODN through
14 an alkylamine, preferably a hexylamine tail, as shown by the partial
15 structure below.

16 $5' \text{ or } 3'-\text{OPO}(\text{OH})-\text{O}-(\text{CH}_2)_6-\text{CO}-(\text{CH}_2)_3-\text{C}_6\text{H}_4-\text{N}-(\text{CH}_2\text{CH}_2\text{Cl})_2$

17 Preferably, the cross-linking function is covalently attached to a
18 nucleotide unit which is internal on the ODN.

19 Sequence specific binding of the ODN to a double stranded DNA
20 or DNA fragment and cross-linking to one DNA strand occurs in
21 accordance with this aspect of the invention based on a "4-letter"
22 Watson-Crick type recognition motif. It has been found however that in
23 vitro a recombinase enzyme is needed for the binding and cross-linking
24 to occur. The recombinase enzyme promotes binding of the ODN to
25 the dsDNA as a triplex. In vivo, recombinase enzymes are virtually

1 ubiquitous and the ODNs in accordance with this aspect of the
2 invention undergo triplex formation and resultant cross-linking due to
3 the presence of the indigenous recombinase enzyme in the cell. The
4 invention however is not limited by the specific nature or origin of the
5 recombinase enzyme, recombinases from single cell organisms as well as
6 from cells of human or mammalian origin are capable of functioning
7 within the invention. Because binding and cross-linking of the ODN to
8 double stranded DNA occurs on the basis of the full "4-letter" Watson
9 Crick recognition motif, this aspect of the invention provides a still
10 broader basis for therapeutic application and as a sequence specific
11 probe (for example for gene mapping) of double stranded DNA than
12 the previously described aspect of the invention wherein the binding of
13 the ODN to the dsDNA is based on Hoogsteen or reverse Hoogsteen
14 pairing.

15 Since the action of a recombinase enzyme is necessary in
16 accordance with this aspect of the invention, the ODNs designed in
17 accordance with this aspect include sugar moieties in their nucleotide
18 units which are compatible with recognition by the recombinase
19 enzyme. Preferably the ODNs in accordance with this aspect comprise
20 2'-deoxyribonucleotides and their isosteric equivalents, 2'-Q-alkyl
21 ribonucleotides (alkyl of C₁ - C₆ carbons) and
22 2'-deoxy-2-fluororibonucleotides.

23 As in the previously described aspect of the invention, target
24 sequences of dsDNA can be deleterious structural genes and associated
25 up-stream and down-stream regulatory control sequences the

1 deactivation of which brings about a therapeutically beneficial result.
2 Target sequences also include genes of invading organisms, such as
3 viruses, fungi, parasites, bacteria and tumor cells. The regulatory
4 sequences may be involved in either transcription or replication. The
5 anti-gene ODN of the invention is determined and designed according
6 to the target DNA sequence chosen for alteration of function. The
7 ODN has a sequence which is homologous (or substantially homologous)
8 to the target sequence in one of the two strands of the chosen target
9 DNA. It follows from the foregoing that the sequence of the ODN
10 which is "homologous" to one strand of the dsDNA or fragment
11 thereof, is complementary in the Watson Crick sense to the other strand
12 of the dsDNA, or fragment thereof. ODNs of this embodiment or
13 aspect of the invention can be used for diagnostic, analytical,
14 "gene-mapping" and like purposes substantially as described above for
15 the second embodiment or aspect of the invention. The advantage of
16 this embodiment is that it operates in a "four-letter" Watson Crick
17 recognition mode.

18 It is an important feature or discovery in accordance with this
19 aspect of the invention that the entire ODN does not need to be
20 homologous (or complementary) to the dsDNA or fragment thereof, but
21 there must be at least approximately 26, and preferably at least
22 approximately 30 nucleotide units in a continuous sequence in the ODN
23 which are homologous (or substantially homologous) to the matching
24 sequence of the dsDNA (or fragment thereof). Moreover, the
25 cross-linking function must be within or attached to an end of the

1 continuous sequence of approximately 26, or more, homologous (or
2 substantially homologous) nucleotide units.

3 The ability of the ODNs in accordance with the present invention
4 to bind to dsDNA (or fragment thereof) and cross-link therewith,
5 provided the above-noted conditions are met, is demonstrated by the
6 following experimental examples.

7 **Experimental Examples of Cross-linking ODNs to Double Stranded**
8 **DNA In Vitro Examples**

9 **Materials and Methods**

10 RecA protein was purchased from US Biochemical Corporation
11 (Cleveland, Ohio). The restriction enzymes EcoRI, ScaI, PvuI and AseI
12 were purchased from New England Biolabs (Beverly, MA). Proteinase
13 K was obtained from Boeringer Mannheim Biochemicals (Indianapolis,
14 IN).

15 Short dsDNA fragments (amplicons) 197 and 272bp long were
16 synthesized by standard PCR protocol (Perkin Elmer Cetus, Norwalk,
17 CT) using EcoRI linearized pBR322 plasmid DNA (Promega) as a
18 template. One of the primers for the amplification reaction was
19 chemically phosphorylated during its synthesis to permit selective
20 5'-³²P-end labeling (using T4 Polynucleotide Kinase and [γ -³²P]ATP) of
21 only one strand of the dsDNA product. As a long dsDNA target
22 pGEM-4Z plasmid DNA (Promega) was used after linearization with
23 ScaI restriction endonuclease and subsequent 5'-³²P-end labeling of both
24 strands. All 5'-³²P-end labeled dsDNA substrates were purified by
25 non-denaturing PAGE or agarose gel electrophoresis prior to use to

1 avoid any possible exonuclease or ssDNA contamination.

2 Oligonucleotides were synthesized by standard phosphoramidite
3 chemistry on an Applied Biosystems 394 DNA/RNA Synthesizer and
4 purified by reverse phase HPLC. The chlorambucil reactive moiety was
5 attached to 5-(3-aminopropyl)-2'-deoxyuridine residues in the
6 oligonucleotides or to a 5'-aminohexyl phosphate group by postsynthetic
7 acylation with chlorambucil 2,3,5,6-tetrafluorophenyl ester as described
8 elsewhere above and in the article by Igor V. Kutyavin, Howard B.
9 Gamper, Alexander A. Gall, and Rich B. Mayer, Jr. (1993) J. Amer.
10 Chem. Soc. **115**, 9303, which is incorporated herein by reference.

11 Standard Reaction Conditions. Synaptic complexes were formed
12 by mixing together 100 nM reactive ODN, 2 μ M RecA protein and
13 10-100 nM dsDNA on ice and then increasing the temperature to 37°C.
14 The reactions were conducted in 50 μ L volumes and contained 10 mM
15 tris-acetate buffer (pH 7.5), 50 mM sodium acetate, 12 mM magnesium
16 acetate, 1 mM DTT, 1 mM Γ -S-ATP and 5% glycerol. Alkylation of the
17 dsDNA target by chlorambucil was allowed to go to completion by
18 incubating the reactions for 6 hours at 37°C.

19 Assay of the Modification Products. To detect the sites of
20 crosslinkage on dsDNA the reaction mixtures were diluted two times
21 with buffer containing 0.5% SDS and 200 μ g/mL proteinase K. After 30
22 min incubation at 37°C samples were extracted once with
23 phenol-chloroform, three times with ether and precipitated by ethyl
24 alcohol. To introduce a nick at the positions of alkylated guanosines,
25 DNA pellets were treated with 10% piperidine for 30 min at 95°C.

1 Samples were precipitated again with ethyl alcohol and the DNA was
2 collected by centrifugation, dried and dissolved in 80% formamide
3 containing 0.1% xylene cyanol and bromophenol blue. Samples were
4 analyzed by 8% denaturing PAGE and sites of DNA cleavage were
5 identified by comparison with the products obtained with Maxam and
6 Gilbert reactions in accordance with the teachings of Maxam, A.M., &
7 Gilbert, W. (1977) Prog. Natl. Acad. Sci. U.S.A. **74**, 560, or with marker
8 prepared by restriction endonuclease digestion.

9 **Formula 2** below depicts the nucleotide sequence of 272bp
10 amplicon identical to region 1523-1794 of pGEM-4Z plasmid DNA

11 1 CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT
12 AATAGTTTGC GCAACGTTGT 50
13 51 TGCCATTGCT ACAGGCATCG TGGTGTCACG
14 CTCGTCGTTT GGTATGGCTT 100
15 101 CATTCAAGCTC CGGTTCCCAA CGATCAAGGC
16 GAGTTACATG ATCCCCCATG 150
17 151 TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT
18 CCTCCGATCG TTGTCAGAAG 200
19 201 TAAGTTGGCC GCAGTGTTAT CACTCATGGT
20 TATGGCAGCA CTGCATAATT 250
21 251 CTCTTACTGT CATGCCATCC GT

22 **Formula 2**

23 **Table 1** below summarizes the results of the above-noted in vitro
24 experiments. The Table shows the sequence of the 272bp amplicon in a
25 region of homology with oligonucleotide reagents. Sequences of

1 reactive oligonucleotides are shown below and are numbered 1, 1a and
2 2 - 12 (non-homologous regions are shown in underline type. U
3 denotes 5-(3-aminopropyl)-2'-deoxyuridine residues with attached
4 chlorambucil moiety, 5'Chlb - denotes a chlorambucil moiety attached
5 to the 5'-phosphate of an oligonucleotide through a 6-aminohexyl linker.

6

Table 1

150 200
 5'...CCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCA...3'
 3'...GSGGGTACACACGTTTTTTCGCCAATCGAGGAAGCCAGGAGGCTAGCAACAGTCTTCATTCAACCGGCGT...5'

ODN	Sequence	Length	Cross link #
1	5'-ATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCU'CCGATCGTTGTCAG	50	56
1a	5' Ch1b-ATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCT CCGATCGTTGTCAG	50	-10
1b	5'-ATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCT CCGATCGTTGTCAG	50	-
2	5'-AAAAAGCGGTTAGCTCCTTCGGTCCU'CCGATCGTTGTCAG	40	43
3	5'-TAGCTCCTTCGGTCCU'CCGATCGTTGTCAG	30	38
4	5'-GGTCCU'CCGATCGTTGTCAG	20	1
5	5'-CCACCACATCGCCGCATAACCGATCCCTTCGGTCCU'CCGATCGTTGTCAG	50	2
6	5'-ATGTTGTGCAAAAAAGCGGTTAGCTTTCCTAAGTTU'TTACCTACCACTGA	50	1
7	5'-CCACCACATCGCCGCATAAGTAGCTCCTTCGGTCCU'CCGATCGTTGTCAG	50	6
8	5'-ATGTTGTGCAAAAAAGCGGTTAGCTCCTTCAAGTTU'TTACCTACCACTGA	50	<1
9	5'-AAAAGCGGTTAGCTCCTTCGGTCCU'CCGATCGTTGTCAGAAGTAAGTTG	49	52
10	5'-AAAAGCGGTTAGCTCCTTCGAGCCU'CCACTCGTTGTCAGAAGTAAGTTG	49	2
11	5'-AAAAGCGGTTAGCTCCTTCGACTCU'CTACTCGTTGTCAGAAGTAAGTTG	49	<1
12	5'-AAAAGCGGTTAGCTCCTTCGACTTU'TTACTCGTTGTCAGAAGTAAGTTG	49	<1

As it can be seen from Table 1, ODN 1 has 50 nucleotide units

1 and these are homologous to a matching sequence in the 272 base pair
2 (bp) amplicon. The cross-linking function is attached to a
3 2'-deoxyuridine which is within the homologous sequence. It was found
4 experimentally that ODN 1 binds to the amplicon and cross-links with
5 one of the guanines immediately flanking the adenine to which the
6 modified deoxyuridine bearing the cross-linking agent is base paired.
7 ODN 1a also has 50 nucleotide units homologous to the amplicon, and
8 has the cross-linking function at its 5' end. ODN 1a also binds and
9 cross-links to the amplicon to a guanine which is in the immediate
10 vicinity of the binding site of the 5' end of ODN 1a. ODN 2 is similar
11 to ODN 1 (has an internal U*) but comprises of only 40 homologous
12 nucleotides. ODN 2 also cross-links with the amplicon, and so does
13 ODN 3 which has 30 homologous nucleotides and an internally located
14 cross-linking function (U*). ODN 4 has only 20 homologous nucleotides
15 and an internally located cross-linking function. ODN 4 does not
16 cross-link to the amplicon, or cross-links only very poorly. ODN 5 has
17 50 nucleotide units, but only 25 are homologous to the amplicon. The
18 internal cross-linking agent is within the homologous sequence, and
19 ODN 5 cross-links only very poorly. This demonstrates that more than
20 25 homologous nucleotides are needed for significant cross-linking.
21 ODN 6 has 25 homologous nucleotides but the cross-linking function is
22 not within the homologous region. ODN 6 does not cross-link or
23 cross-links only very poorly. ODN 7 has 30 homologous nucleotides in
24 sequence, and the cross-linking function is in the homologous region.
25 ODN 7 cross-links demonstrably, despite the presence of a sequence of

1 20 non-homologous nucleotides. ODN 8 has 30 homologous and 20
2 non-homologous nucleotides, each in a continuous sequence. The
3 cross-linking agent is in the non-homologous sequence and ODN 8 does
4 not cross-link. ODN 9 has 49 homologous units in sequence and
5 cross-links. ODNs 10 - 12 each have only a few non-homologous
6 nucleotides, but the non-homologous units are located close (next to or
7 within 1 and 2 units respectively) to the nucleotide bearing the
8 cross-linking function. ODNs 10 - 12 do not cross-link, or cross-link
9 only very poorly.

10 In related experiments the ODNs designated ODN 1 and 1a in
11 Table 1 were incubated with the 272 bp amplicon under the above
12 described conditions but in the absence of recombinase enzyme. No
13 cross-linking was observed in these experiments. In still related
14 experiments the efficacy of the cross-linking between internally located
15 cross-linking function (ODN 1 of Table 1) and terminally located
16 cross-linking function (ODN 1a of Table 1) was compared by examining
17 the intensity of the appropriate bands obtained in PAGE
18 electrophoresis, pursuant to the Maxam Gilbert sequencing method:
19 The internally located cross-linking function was found to be
20 approximately 7 times more effective than the terminally located
21 cross-linking function.

22 In Vitro and In Vivo Cross-linking with Human Genomic DNA

23 The ODNs used in these experiments were a 50mer and a 30mer
24 of the following structures:

25 50-mer:

1 GGTTATTTTTGAAGATACGAATTCU*CCAGAGACACAGCAGG
2 ATTTGTCA-HEXANOL

3 30-mer:

4 GAAGATACGAATTCU*CCAGAGACACAGCA-HEXANOL

5 In these structures U* denotes 5-(3-aminopropyl)-2'-deoxyuridine
6 residues with an attached chlorambucil moiety. These ODNs are
7 complementary to the coding strand of a HLA DQB1 allele 0302
8 (nucleotides 815 - 864 and 825 - 854 according to Larhammar, D. et al.
9 (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 7313 - 7317. In the in vitro
10 experiment, cross-linking oligonucleotides were added to the naked
11 human genomic DNA with or without recombinase enzyme (RecA from
12 Escherichia coli).

13 Experimental description

14 Reagents were mixed to give final concentrations of genomic
15 DNA - 40 µg/ml; crosslinking ODN - 5×10^{-7} M; RecA = 2×10^{-6} M;
16 MgCl₂ - 12 mM; ATP gamma-S - 1 mM. In the control reactions the
17 presence of either RecA or oligonucleotides was omitted. The solutions
18 were incubated at 37°C for 3 hours, then deproteinized with Proteinase
19 K/SDS for 30 minutes at 37°C. DNA was recovered by
20 phenol:chloroform extraction and treated with 1M pyrrolidine at 90° C
21 for 30 minutes to cleave the DNA at the crosslinking sites. The DNA
22 was precipitated by ethanol and the Ligation-Mediated PCR reaction
23 was performed as described by Chong-Soon Lee et.al. (Biochemistry
24 1994, 33, 6024-6030) to visualize nicks caused by alkylation at
25 crosslinking sites.

1 The results of this experiment, as observed on PAGE
2 electrophoresis, were that binding of the 50mer and of the 30mer to the
3 matching sequence in the human genomic allele and subsequent
4 cross-linking had occurred, but only when the ODN and the
5 recombinase enzyme were both present in the incubation mixture. This
6 experiment proves site specific alkylation (cross-linking with the ODN)
7 of whole human genomic DNA in vitro with the ODN of the invention
8 that is complementary in the Watson Crick sense to a sequence in the
9 double stranded genomic DNA.

10 In an in vivo cell culture experiment the 50mer and the 30mer
11 ODNs were added to the culture of BSM B-lymphocyte cells under
12 conditions described below. Experimental Description

13 BSM B-lymphocyte cells were grown in a 25 ml flask to a density
14 of 4.5×10^6 cells per ml.

15 Media:

16 500 ml RPMI 1640 with L-glutamine (2mM) (Gibco BRL Cat. No.
17 11875-036)

18 50 ml of HI-FCS (fetal calf serum: Gibco BRL Cat. No. 26140, heat
19 inactivated 30' at 55°C)

20 5 ml of 100X Penn/Strep (Gibco BRL Cat. No. 15070-022)

21 5 ml of 200 mM L-Glutamine (Gibco BRL Cat. No. 25030-024)

22 5 ml of 100X Sodium Pyruvate (11 mg/ml filter sterilized) of 1 M
23 HEPES, pH 7.3 (Gibco BRL Cat. No. 15630-023).

24 For each treatment, 2 ml was taken from a BSM cell flask (25ml)
25 and was spun 5 minutes at 1,200 rpm, then resuspended in:

1	ODN	ODN	μ l serum-	μ l ODN (from water
2	NAME	Conc (μ M)	free media	10^{-4} M in water)
3	24.01(50-mer)	0	160	0 40
4	24.01(50-mer)	1	160	2 38
5	24.01(50-mer)	10	160	20 20
6	24.01(50-mer)	50	160	20 (from 20
7				5×10^{-4} M)

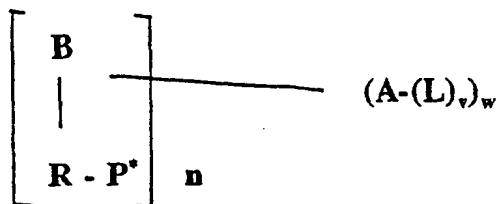
8 Note: Serum free media is identical to the above media except for the
 9 absence of 50 ml of HI-FCS (Gibco BRL Cat. No. 26140, heat
 10 inactivated 30 min at 55°C).

11 Each sample was incubated for 3.5 hours at 37° C and 5% CO₂ in
 12 a 48-well microtiter plate. Cells were transferred to 1.5 ml plastic
 13 centrifuge tubes, pelleted 5' at 2,000 rpm, washed twice with 500 μ l PBS
 14 and deproteinized with Proteinase K/SDS overnight at 37° C. DNA was
 15 recovered by phenol:chloroform extraction and Rnase A digestion and
 16 treated with 1M pyrrolidine at 90° C for 30 min to cleave DNA at the
 17 crosslinking sites. Pyrrolidine was removed by ethanol precipitation and
 18 the Ligation-Mediated PCR reaction was performed as described by
 19 Chong-Soon Lee et. al. (supra) to visualize nicks caused by alkylation.

20 The foregoing experiments showed that the 50mer and the 30mer
 21 ODN sequence specifically bound to and alkylated (cross-linked) the
 22 0302 allele in the human genomic DNA.

23 In light of the foregoing, a general structure of the
 24 oligonucleotides of the present invention is given by **Formula 3**

70



Formula 3

where **B-R-P*** represents a nucleotide building block of the oligonucleotide which may optionally bear a reporter group or may optionally include a radioactive label. **B-R-P*** includes intermediate nucleotide units and the 5'- and 3' terminal nucleotide units. Specifically, **B** represents a heterocyclic base component of the nucleotide, **R** represents a sugar moiety which forms a pyranose or furanose ring, or an isosteric analog thereof, and **P*** represents a phosphate group including a phosphate monoester, phosphate diester or phosphate triester group, or **P*** represents a monothioate or dithioate analog of said phosphate groups. **P*** further includes the above-noted phosphate, phosphothioate or phosphodithioate groups in internucleotidic linkages, and also at the 5' and 3' terminus of the oligonucleotide. The $(\text{A}-(\text{L})_v)_w$ grouping forms an electrophilic alkylating group such that **L** is a leaving group and **A-(L)_v** is inert under conditions of hybridization with the target sequence of DNA or RNA, in the sense that the group **A-L** reacts only after hybridization with the target sequence. **n** is an integer with the values between 5 and approximately 3000; **v** is 1 or 2; and **w** is between 1 - 10, and the oligonucleotide includes a sequence which is complementary in the Watson Crick, Hoogsteen or reverse Hoogsteen sense to a target sequence of single or double stranded DNA or single stranded RNA.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: MicroProbe Corporation
Bothell, WA 98021
- (ii) TITLE OF INVENTION: CROSSLINKING OLIGONUCLEOTIDES
- (iii) NUMBER OF SEQUENCES: 28
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Klein & Szekeres
 - (B) STREET: 4199 Campus Drive, Suite 700
 - (C) CITY: Irvine
 - (D) STATE: CA
 - (E) COUNTRY: USA
 - (F) ZIP: 92715
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: PCT
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/485,611
 - (B) FILING DATE: 07-JUN-1995
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/226,949
 - (B) FILING DATE: 27-JUN-1994
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/011,482
 - (B) FILING DATE: 26-JAN-1993
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/334,490
 - (B) FILING DATE: 04-NOV-1994
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/049,807
 - (B) FILING DATE: 20-APR-1993
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/353,857
 - (B) FILING DATE: 18-MAY-1989

72

- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/250,474
 - (B) FILING DATE: 28-SEP-1988
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/178,733
 - (B) FILING DATE: 07-JAN-1994
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/748,138
 - (B) FILING DATE: 21-AUG-1991
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/353,857
 - (B) FILING DATE: 18-MAY-1989
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Szekeres, Gabor L.
 - (B) REGISTRATION NUMBER: 28,675
 - (C) REFERENCE/DOCKET NUMBER: 491-11-CIP
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 714-854-5502
 - (B) TELEFAX: 714-854-4897

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGGATGTUC CTTC

14

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGACAGCACA GAATTCGAAG GAACATCCAG

30

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACCGTCCTTG ACACGATGGA CTCC

24

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTCCAUCGTG TCAAG

15

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

NAGAGGAGAA AGGAGAGAGN

20

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATATAAGGAG AGAGGAAAGA GGAGACAAA

29

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTGTGGTGGT YGTGTYGTGG TGGG

24

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGAGGAGCA GAGGAGGAGG AGAA

24

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTTTCTTTTY GGGGGTN

17

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTTTAAAAG AAAAGGGGGG ACTGG

25

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

NCTTTCCTCT CTTTTCGCCN

20

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AAATACTGGG AGAAAGGAGA GAAAAGGGGA CCCAACGTAT

40

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 272 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT AATAGTTTGC GCAACGTTGT TGCCATTGCT 60
ACAGGCATCG TGGTGTACAG CTCGTCGTTT GGTATGGCTT CATTACAGCTC CGGTTCCCAA 120
CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT 180
CCTCCGATCG TTGTCAGAAG TAAGTTGGCC GCAGTGTTAT CACTCATGGT TATGGCAGCA 240
CTGCATAATT CTCTTACTGT CATGCCATCC GT 272

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATGTTGTGCA AAAAAGCGGT TAGCTCCTTC GGTCCUCCGA TCGTTGTCAG

50

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATGTTGTGCA AAAAAGCGGT TAGCTCCTTC GGTCCUCCGA TCGTTGTCAG

50

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AAAAAGCGGT TAGCTCCTTC GGTCCUCCGA TCGTTGTCAG

40

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TAGCTCCTTC GGTCCUCCGA TCGTTGTCAG

30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGTCCUCCGA TCGTTGTCAG

20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCACCACATC GCCGCATAAC CGATCCCTTC GGTCCUCCGA TCGTTGTCAG

50

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATGTTGTGCA AAAAAGCGGT TAGCTTTCCT AACTTUTTAC CTACCACTGA

50

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCACCACATC GCCGCATAAC TAGCTCCTTC GGTCCUCCGA TCGTTGTCAG

50

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGTTGTGCA AAAAAGCGGT TAGCTCCTTC AACTTUTTAC CTACCACTGA

50

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AAAAGCGGTT AGCTCCTTCG GTCCUCCGAT CGTTGTCAGA AGTAAGTTG

49

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AAAAGCGGTT AGCTCCTTCG ACCCUCCACT CGTTGTCAGA AGTAAGTTG

49

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AAAAGCGGTT AGCTCCTTCG ACTCUCTACT CGTTGTCAGA AGTAAGTTG

49

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AAAAGCGGTT AGCTCCTTCG ACTTUTTACT CGTTGTCAGA AGTAAGTTG

49

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GGTTATTTTT GAAGATACGA ATTCUCCAG AGACACAGCA GGATTTGTCA

50

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

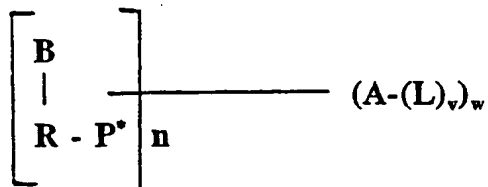
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GAAGATACGA ATTCUCCAG AGACACAGCA

30

WHAT IS CLAIMED IS:

1. An oligonucleotide of the formula



where **B-R-P*** represents a nucleotide building block of the oligonucleotide which may optionally bear a reporter group or may optionally include a radioactive label; **B** in said **B-R-P*** represents a heterocyclic base component of the nucleotide, **R** in said **B-R-P*** represents a sugar moiety which forms a pyranose or furanose ring, or an isosteric analog thereof, and **P*** in said **B-R-P*** represents a phosphate group including a phosphate monoester, phosphate diester or phosphate triester group, or **P*** represents a monothioate or dithioate analog of said phosphate groups;

$(\text{A}-(\text{L})_v)_w$ represents an electrophilic alkylating group wherein **L** is an electrophilic leaving group, **A** is a group that covalently links **L** to the oligonucleotide and $\text{A}-(\text{L})_v$ is inert under conditions of hybridization with the target sequence of DNA or RNA, and $\text{A}-(\text{L})_v$ reacts only after hybridization with the target sequence;

n is an integer with the values between 5 and approximately 3000,

v is 1 or 2;

w is between 1 - 10, and the oligonucleotide includes a sequence which is complementary in the Watson Crick, Hoogsteen or reverse Hoogsteen sense to a target sequence of single or double stranded DNA.

1 or single stranded RNA.

2 2. The oligonucleotide of Claim 1 wherein the oligonucleotide
3 has 5 to approximately 300 nucleotide units, and includes a sequence
4 which is complementary in the Watson Crick sense to a target sequence
5 in RNA, single or double stranded DNA.

6 3. The oligonucleotide of Claim 2 wherein the R group
7 represents β -2-deoxyribofuranose, β -ribofuranose,
8 β -2-O-methoxyribofuranose, and β -2-deoxy-2-fluororibofuranose.

9 4. The oligonucleotide of Claim 2 wherein the $(A-(L)_v)_w$ is
10 covalently attached to a heterocyclic base component of the
11 oligonucleotide.

12 5. The oligonucleotide of Claim 4 wherein the $(A-(L)_v)_w$ group
13 is selected from the groups consisting of

14 $-(CH_2)_q - Y - (CH_2)_m - L$,

15 $-(CH_2)_q - NH - CO - (CH_2)_m - (X)_{n'} - N(R_1) - (CH_2)_p - L$, and

16 $-(CH_2)_q - O - (CH_2)_q - NH - CO - (CH_2)_m - (X)_{n'} - N(R_1) - (CH_2)_p - L$

17 where each of m and q is independently 0 to 8, inclusive, q' is 3
18 to 7 inclusive, q'' is 1 to 7 inclusive,

19 Y is a linking group derived from a bifunctional molecule having
20 a hydrocarbyl backbone and having at each end a functionality selected
21 from $-NH_2$, $-OH$, SH , $-COOH$ and $C \equiv CH$, and

22 X is phenyl, or phenyl substituted with chloro, bromo, lower alkyl
23 or lower alkoxy groups, n' is 0 or 1, p is an integer from 1 to 6, and R_1
24 is H, lower alkyl or $(CH_2)_p - L$.

25 6. The oligonucleotide of Claim 5 where the $(A-(L)_v)_w$ group is

1 $-(\text{CH}_2)_q - \text{Y} - (\text{CH}_2)_m - \text{L}$.

2 7. The oligonucleotide of Claim 6 where the $-(\text{CH}_2)_q - \text{Y} -$
 3 $(\text{CH}_2)_m - \text{L}$ group is selected from 3-iodoacetamidopropyl,
 4 3-(4-bromobutyramido)propyl, 4-iodoacetamidobutyl and
 5 4-(4-bromobutyramido)butyl.

6 8. The oligonucleotide of Claim 6 bearing a radioactive label.

7 9. The oligonucleotide of Claim 1 wherein the L group is
 8 selected from a group consisting of chloro, bromo, iodo, $\text{SO}_2\text{R}'''$, and
 9 $\text{S}^+\text{R}'''\text{R}''''$, where each of R''' and R'''' is independently C_{1-6} alkyl or aryl or
 10 R''' and R'''' together form a C_{1-6} alkylene bridge.

11 10. The oligonucleotide of Claim 1 wherein the oligonucleotide
 12 has 5 to approximately 300 nucleotide units, and includes a sequence
 13 which is complementary in the Hoogsteen or reverse Hoogsteen sense
 14 to a target sequence in double stranded DNA, and wherein in the
 15 definition of $(\text{A}-(\text{L})_v)_w$

16 when w is 2 to 10 then v is 1 or 2, and

17 when w is 1 then v is 2, whereby the oligonucleotide includes at
 18 least two alkylating functions.

19 11. The oligonucleotide of Claim 10 wherein the cross-linking
 20 agents $(\text{A}-(\text{L})_v)_w$ are attached to the heterocyclic bases.

21 12. The oligonucleotide of Claim 11 wherein
 22 $(\text{A}-(\text{L})_v)_w$ is a group selected from the groups consisting of

23 $-(\text{CH}_2)_q - \text{Y} - (\text{CH}_2)_m - \text{L}$, $(\text{CH}_2)_q - \text{CO} - \text{CH}_2 - \text{L}$,

24 $-(\text{CH}_2)_q - \text{NH} - \text{CO} - (\text{CH}_2)_m - (\text{X})_{n'} - \text{N}(\text{R}_1) - (\text{CH}_2)_p - \text{L}$, and

25 $-(\text{CH}_2)_q - \text{O} - (\text{CH}_2)_q - \text{NH} - \text{CO} - (\text{CH}_2)_m - (\text{X})_{n'} - \text{N}(\text{R}_1) - (\text{CH}_2)_p - \text{L}$

1 where each of m and q is independently 0 to 8, inclusive, q' is 3 to 7
 2 inclusive, q'' is 1 to 7 inclusive,

3 Y is a linking group derived from a bifunctional molecule having
 4 a hydrocarbyl backbone and having at each end a functionality selected
 5 from $-NH_2$, $-OH$, SH , $-COOH$ and $C\equiv CH$, and

6 X is phenyl, or phenyl substituted with chloro, bromo, lower alkyl
 7 or lower alkoxy groups, n' is 0 or 1, p is an integer from 1 to 6, and R_1
 8 is H, lower alkyl or $(CH_2)_p-L$.

9 13. The oligonucleotide of Claim 12 wherein the cross-linking
 10 agents $(A-(L)_v)_w$ are attached to the 5-position of uracil bases.

11 14. The oligonucleotide of Claim 13 wherein the
 12 $(A-(L)_v)_w$ group is selected from the group consisting of
 13 $-(CH_2)_3O(CH_2)_2NHCO(CH_2)_3-C_6H_4-N-[CH_2CH_2Cl]_2$ and
 14 $-(CH_2)_3NHCO(CH_2)_3-C_6H_4-N-[CH_2CH_2Cl]_2$.

15 15. The oligonucleotide of Claim 10 wherein the
 16 $(A-(L)_v)_w$ group is equivalent in length to a normal alkyl chain of
 17 approximately 2 to 50 carbons.

18 16. The oligonucleotide of Claim 10 wherein the cross-linking
 19 agents $(A-(L)_v)_w$ are attached to phosphate groups.

20 17. The oligonucleotide of Claim 16 which has a 3' and a 5'
 21 phosphate terminus, and wherein the cross linking agents $(A-(L)_v)_w$ are
 22 attached to the 3' and 5' phosphate termini.

23 18. The oligonucleotide of Claim 17 wherein
 24 $(A-(L)_v)_w$ is a group selected from the groups consisting of
 25 $-(CH_2)_q - Y - ((CH_2)_m - L, (CH_2)_q - CO - CH_2 - L, - (CH_2)_q - NH$

1 - CO - (CH₂)_m - (X)_{n'} - N(R₁) - (CH₂)_p - L, and

2 -(CH₂)_{q'} - O - (CH₂)_q - NH - CO - (CH₂)_m - (X)_{n'} - N(R₁) - (CH₂)_p - L

3 where each of m and q is independently 0 to 8, inclusive, q' is 3
4 to 7 inclusive, q'' is 1 to 7 inclusive,

5 Y is a linking group derived from a bifunctional molecule having
6 a hydrocarbyl backbone and having at each end a functionality selected
7 from -NH₂, -OH, SH, -COOH and C≡CH, and

8 X is phenyl, or phenyl substituted with chloro, bromo, lower alkyl
9 or lower alkoxy groups, n' is 0 or 1, p is an integer from 1 to 6, and R₁
10 is H, lower alkyl or (CH₂)_p - L.

11 19. The oligonucleotide of Claim 10 wherein the (A-(L))_w
12 group is selected from the group consisting of

13 -(CH₂)₃O(CH₂)₂NHCO(CH₂)₃-C₆H₄-N-[CH₂CH₂Cl]₂

14 -(CH₂)₃NHCO(CH₂)₃-C₆H₄-N-[CH₂CH₂Cl]₂, and

15 -(CH₂)₆NHCO(CH₂)₃-C₆H₄-N-[CH₂CH₂Cl]₂.

16 20. The oligonucleotide of Claim 10 wherein the L group is
17 selected from a group consisting of chloro, bromo, iodo, SO₂R''', and
18 S⁺R''R''', where each of R''' and R''' is independently C₁₋₆alkyl or aryl or
19 R''' and R''' together form a C₁₋₆alkylene bridge.

20 21. The oligonucleotide of Claim 1 which includes a continuous
21 sequence of at least approximately 26 nucleotide units which are
22 complementary in a Watson Crick sense to a target sequence of double
23 stranded DNA and wherein the (A-(L))_w group is covalently attached to
24 the continuous sequence that is complementary to the target sequence,
25 and wherein the R group in each oligonucleotide unit is selected from

1 2-deoxyribofuranosyl, and 2-O-alkylribofuranosyl,

2 2-deoxy-2-fluororibofuranosyl.

3 22. The oligonucleotide of Claim 21 having no more than
4 approximately 300 nucleotide units.

5 23. The oligonucleotide of Claim 22 having no more than
6 approximately 60 nucleotide units.

7 24. The oligonucleotide of Claim 21 wherein the $(A-(L))_w$
8 group is covalently attached to a nucleotide unit which is internal within
9 the continuous sequence that is complementary to the target sequence
10 of double stranded DNA.

11 25. The oligonucleotide of Claim 21 wherein
12 $(A-(L))_w$ is a group selected from the groups consisting of

13 $-(CH_2)_q - Y - (CH_2)_m - L$, $(CH_2)_q - CO - CH_2 - L$

14 $-(CH_2)_q - NH - CO - (CH_2)_m - (X)_n - N(R_1) - (CH_2)_p - L$, and

15 $-(CH_2)_q - O - (CH_2)_q - NH - CO - (CH_2)_m - (X)_n - N(R_1) - (CH_2)_p - L$

16 where each of m and q is independently 0 to 8, inclusive, q' is 3
17 to 7 inclusive, q'' is 1 to 7 inclusive,

18 Y is a linking group derived from a bifunctional molecule having
19 a hydrocarbyl backbone and having at each end a functionality selected
20 from $-NH_2$, $-OH$, SH , $-COOH$ and $C \equiv CH$, and

21 X is phenyl, or phenyl substituted with chloro, bromo, lower alkyl
22 or lower alkoxy groups, n' is 0 or 1, p is an integer from 1 to 6, and R_1
23 is H, lower alkyl or $(CH_2)_p - L$.

24 26. The oligonucleotide of Claim 25 wherein the
25 $(A-(L))_w$ group is selected from the group consisting of

- 1 $-(\text{CH}_2)_3\text{O}(\text{CH}_2)_2\text{NHCO}(\text{CH}_2)_3-\text{C}_6\text{H}_4-\text{N}-[\text{CH}_2\text{CH}_2\text{Cl}]_2,$
 2 $-(\text{CH}_2)_3\text{NHCO}(\text{CH}_2)_3-\text{C}_6\text{H}_4-\text{N}-[\text{CH}_2\text{CH}_2\text{Cl}]_2,$ and
 3 $-(\text{CH}_2)_6\text{NHCO}(\text{CH}_2)_3-\text{C}_6\text{H}_4-\text{N}-[\text{CH}_2\text{CH}_2\text{Cl}]_2.$

4 27. The oligonucleotide of Claim 25 wherein the cross-linking
 5 agents $(\text{A}-(\text{L})_v)_w$ are attached to the 5-position of uracil bases.

6 28. The oligonucleotide of Claim 27 wherein the
 7 $(\text{A}-(\text{L})_v)_w$ group is selected from the group consisting of
 8 $-(\text{CH}_2)_3\text{O}(\text{CH}_2)_2\text{NHCO}(\text{CH}_2)_3-\text{C}_6\text{H}_4-\text{N}-[\text{CH}_2\text{CH}_2\text{Cl}]_2$ and
 9 $-(\text{CH}_2)_3\text{NHCO}(\text{CH}_2)_3-\text{C}_6\text{H}_4-\text{N}-[\text{CH}_2\text{CH}_2\text{Cl}]_2.$

10 29. The oligonucleotide of Claim 25 wherein the $-(\text{CH}_2)_q - \text{Y} -$
 11 $(\text{CH}_2)_m - \text{L}$ group is selected from 3-iodoacetamidopropyl,
 12 3-(4-bromobutyramido)propyl, 4-iodoacetamidobutyl and
 13 4-(4-bromobutyramido)butyl.

14 30. The oligonucleotide of Claim 21 wherein the L group is
 15 selected from a group consisting of chloro, bromo, iodo, $\text{SO}_2\text{R}'''$, and
 16 $\text{S}^+\text{R}'''\text{R}''''$, where each of R''' and R'''' is independently C_{1-6} alkyl or aryl or
 17 R''' and R'''' together form a C_{1-6} alkylene bridge.

18 31. The oligonucleotide of Claim 21 wherein the cross-linking
 19 agents $(\text{A}-(\text{L})_v)_w$ are attached to phosphate groups.

20 32. The oligonucleotide of Claim 31 wherein
 21 $(\text{A}-(\text{L})_v)_w$ is a group selected from the groups consisting of

- 22 $-(\text{CH}_2)_q - \text{Y} - (\text{CH}_2)_m - \text{L}, (\text{CH}_2)_q-\text{CO}-\text{CH}_2-\text{L},$
 23 $-(\text{CH}_2)_q - \text{NH} - \text{CO} - (\text{CH}_2)_m - (\text{X})_{n'} - \text{N}(\text{R}_1)-(\text{CH}_2)_p-\text{L},$ and
 24 $-(\text{CH}_2)_{q'}-\text{O}-(\text{CH}_2)_{q'}-\text{NH}-\text{CO}-(\text{CH}_2)_m - (\text{X})_{n'} - \text{N}(\text{R}_1)-(\text{CH}_2)_p-\text{L}$

25 where each of m and q is independently 0 to 8, inclusive, q' is 3

1 to 7 inclusive, q'' is 1 to 7 inclusive,

2 Y is a linking group derived from a bifunctional molecule having
3 a hydrocarbyl backbone and having at each end a functionality selected
4 from $-NH_2$, $-OH$, SH , $-COOH$ and $C\equiv CH$, and

5 X is phenyl, or phenyl substituted with chloro, bromo, lower alkyl
6 or lower alkoxy groups, n' is 0 or 1, p is an integer from 1 to 6, and R_1
7 is H, lower alkyl or $(CH_2)_p-L$.

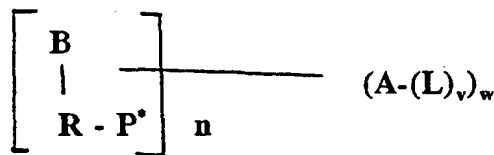
8 33. The oligonucleotide of Claim 32 wherein the $(A-(L)_v)_w$
9 group is selected from the group consisting of

10 $-(CH_2)_3O(CH_2)_2NHCO(CH_2)_3-C_6H_4-N-[CH_2CH_2Cl]_2$
11 $-(CH_2)_3NHCO(CH_2)_3-C_6H_4-N-[CH_2CH_2Cl]_2$, and
12 $-(CH_2)_6NHCO(CH_2)_3-C_6H_4-N-[CH_2CH_2Cl]_2$.

13 34. The oligonucleotide of Claim 32 wherein the $(A-(L)_v)_w$
14 group is $-(CH_2)_6NHCO(CH_2)_3-C_6H_4-N-[CH_2CH_2Cl]_2$.

15 35. A process for cross-linking with a target sequence in double
16 stranded DNA, the process comprising:

17 contacting in the presence of a recombinase enzyme the double
18 stranded DNA having the target sequence with an oligonucleotide of
19 the formula



1 where **B-R-P*** represents a nucleotide building block of the
2 oligonucleotide which may optionally bear a reporter group or may
3 optionally include a radioactive label; **B** in said **B-R-P*** represents a
4 heterocyclic base component of the nucleotide, **R** in said **B-R-P***
5 represents a sugar moiety selected from 2-deoxy-D-ribofuranose or
6 2-deoxy-2-fluoro-D-ribofuranose, and **P*** in said **B-R-P*** represents a
7 phosphate group including a phosphate monoester, phosphate diester
8 or phosphate triester group, or **P*** represents a monothioate or dithioate
9 analog of said phosphate groups;

10 $(A-(L)_v)_w$ represents an electrophilic alkylating group wherein **L**
11 is an electrophilic leaving group, **A** is a group that covalently links **L** to
12 the oligonucleotide and **A-L** is inert under conditions of hybridization
13 with the target sequence of double stranded DNA, and **A-L** reacts only
14 after hybridization with the target sequence;

15 **n** is an integer with the values between 5 and approximately 3000,

16 **v** is 1 or 2;

17 **w** is between 1 - 10,

18 and the oligonucleotide includes a continuous sequence of at least
19 approximately 26 nucleotide units which sequence is complementary in
20 the Watson Crick sense to the a target sequence in the double stranded
21 DNA, and wherein the $(A-(L)_v)_w$ group is covalently attached to the
22 continuous sequence that is complementary to the target sequence.

23 36. The process of Claim 35 which is performed in vitro.

24 37. The process of Claim 35 wherein the oligonucleotide has
25 no more than approximately 300 nucleotide units.

1 38. The oligonucleotide of Claim 27 having no more than
2 approximately 60 nucleotide units.

3 39. The process of Claim 35 wherein the $(A-(L)_v)_w$ is a group
4 selected from the groups consisting of

5 $-(CH_2)_q - Y - (CH_2)_m - L$, $(CH_2)_q - CO - CH_2 - L$,
6 $-(CH_2)_q - NH - CO - (CH_2)_m - (X)_{n'} - N(R_1) - (CH_2)_p - L$, and
7 $-(CH_2)_q - O - (CH_2)_{q'} - NH - CO - (CH_2)_m - (X)_{n'} - N(R_1) - (CH_2)_p - L$

8 where each of m and q is independently 0 to 8, inclusive, q' is 3
9 to 7 inclusive, q'' is 1 to 7 inclusive,

10 Y is a linking group derived from a bifunctional molecule having
11 a hydrocarbyl backbone and having at each end a functionality selected
12 from $-NH_2$, $-OH$, SH , $-COOH$ and $C \equiv CH$, and

13 X is phenyl, or phenyl substituted with chloro, bromo, lower alkyl
14 or lower alkoxy groups, n' is 0 or 1, p is an integer from 1 to 6, and R_1
15 is H, lower alkyl or $(CH_2)_p - L$.

16 40. The process of Claim 39 wherein the $(A-(L)_v)_w$ group is
17 selected from the group consisting of

18 $-(CH_2)_3O(CH_2)_2NHCO(CH_2)_3-C_6H_4-N-[CH_2CH_2Cl]_2$
19 $-(CH_2)_3NHCO(CH_2)_3-C_6H_4-N-[CH_2CH_2Cl]_2$, and
20 $-(CH_2)_6NHCO(CH_2)_3-C_6H_4-N-[CH_2CH_2Cl]_2$.

21 41. The process of Claim 39 wherein the $(A-(L)_v)_w$ group is
22 covalently attached to a nucleotide unit which is internal within the
23 continuous sequence that is complementary to the target sequence of
24 double stranded DNA.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/09551

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	W0,A,94 17092 (MICROPROBE CORP) 4 August 1994 see the whole document ---	1-41
X	W0,A,93 03736 (MICROPROBE CORP) 4 March 1993 see the whole document ---	1-41
X	W0,A,90 14353 (MICROPROBE CORP) 29 November 1990 see the whole document -----	1-41

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

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- *O* document referring to an oral disclosure, use, exhibition or other means
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- *&* document member of the same patent family

Date of the actual completion of the international search

4 November 1996

Date of mailing of the international search report

28. 11. 96

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/09551

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9417092	04-08-94	AU-A- 6296294	15-08-94
WO-A-9303736	04-03-93	EP-A- 0661979 JP-T- 6509945	12-07-95 10-11-94
WO-A-9014353	29-11-90	EP-A- 0472648 JP-T- 4507402	04-03-92 24-12-92